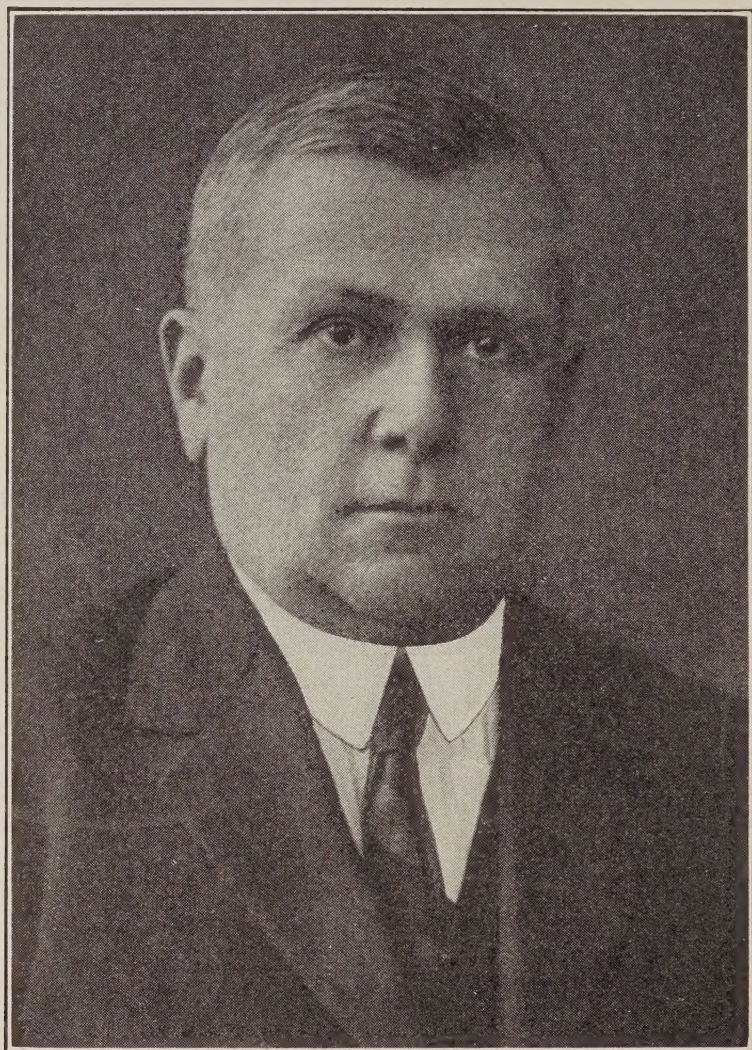


KOSTYCHEV'S CHEMICAL
PLANT PHYSIOLOGY



Frontispiece

Doctor S. Kostychev

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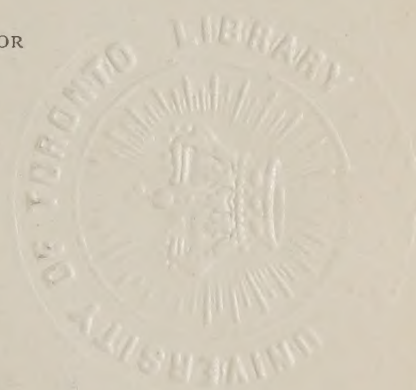
KOSTYCHEV'S CHEMICAL PLANT PHYSIOLOGY

AUTHORIZED EDITION IN ENGLISH
WITH EDITORIAL NOTES

WRITTEN AND REVISED BY
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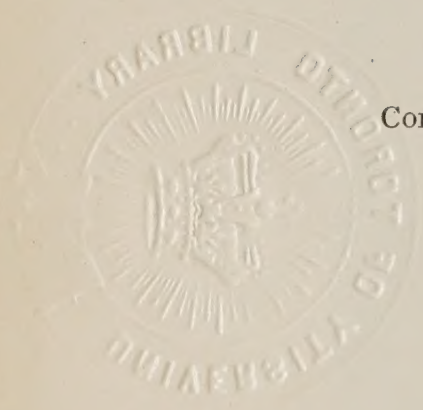


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AUTHOR'S PREFACE

Every exact science passes through a certain stage of important transformation which conditions its further evolution. This kind of a change took place in mathematics and mechanics during the years when Euler, Lagrange, Laplace and other makers of mathematical analysis were active. General chemistry was transformed by the discoveries of Lavoisier. For organic chemistry, the middle of the 19th century, when the first basic syntheses were effected and when the coherent classification of the organic compounds originated, was an epoch of "storm and pressure."

For plant physiology, this period began very gradually. The times of heroic deeds are past and the changes in the exact sciences occur nowadays without upsetting the fundamental conceptions as Lavoisier did. Nevertheless, there have been rapid changes in single branches of science even in our time. The latest achievements of physical and biological chemistry are responsible for the recent notable advances in chemical plant physiology. They made it possible to work out the problems which form the very foundation of physiology and which were incapable of being attacked twenty-five years ago. The nature of the most important biochemical processes first became the subject of experimental investigation in the 20th century. This was really the transformation of chemical plant physiology. The magnitude of the change in thought and of the body of facts brought out during the past twenty-five years in the field of chemical plant physiology, may be seen by comparing the contents of the first sections of this book with the corresponding chapters of the "Pflanzenphysiologie" by W. Pfeffer (1897). This comparison shows that from the science of that time only a barely recognizable framework has been retained and that countless new problems have emerged.

Even in the latest text books, the rapid advances of chemical plant physiology have not always been given enough attention. It would appear to be very important that this young secondary growth of plant physiology be equal to the problems which it faces. In the first place, we must overcome the chemical dilettantism which in former times was peculiar to all biology to a greater or lesser degree and which occasionally appears even now. The present worker in the field of chemical plant physiology must be a trained chemist as well. Failure to meet this requirement might ultimately result in monopoly of the most important problems of plant biochemistry by non-botanists, a situation which should be avoided since non-botanists do not always possess the necessary preparation in other respects.

This book aims especially at emphasis upon the all important chemical aspects of the physiological processes. Hence it is based chiefly upon principles

of methodology. To be sure, the methods are brought out only from the theoretical standpoint but the citations include the necessary references to the actual experimental work. The historical method of approach does not enter into this book so much as it does in others. That is to say, plant physiology has now become too extensive a science to be treated historically. Only two sections—photosynthesis and fermentations—contain brief historical sketches in consideration of the instructive data contained in them. Each part of the book begins with an introductory chapter which contains brief expositions of the physico-chemical principles of those sections of plant physiology that are considered.

The writer assumes that the reader is already familiar with the fundamental principles of organic chemistry. The contents of the first part of this book also provides a striking demonstration that a certain amount of chemical preparation is indispensable for plant physiologists. The book is made as brief as possible. To what extent it has been successful, by the use of a somewhat summarized exposition to prevent misunderstandings, the reader may judge.

The author wishes to be advised by letter of any omissions or errors. All such suggestions will be thankfully received.

S. KOSTYCHEV.

Leningrad.

EDITOR'S NOTE

This book is an up-to-date rendition of a volume which appeared in German in 1926 as part one of the "Lehrbuch der Pflanzenphysiologie" by Dr. S. Kostychev of Leningrad. The first volume was entitled "Chemische Physiologie" with the implication that the second volume would deal with the physical aspects of plant physiology. The second volume has not yet appeared but the value of the first warranted its translation into English for the benefit of a large body of students who cannot afford the time required to read extensively in the German language.

Dr. Kostychev's treatment of the chemical aspects of plant physiology—a subject sometimes called "biochemistry of plants"—is so direct and thorough that my efforts have been limited almost entirely to the preparation of an accurate and fairly close translation. Points or citations for which I am responsible are indicated by brackets or contained in signed foot-notes. The number of such additions is relatively small and my one purpose has been to supplement rather than to contradict the author's points by the use of facts which have come to my attention. No attempt has been made to collect references to all work not cited by the author although some attention was given to the provision of references to papers in English which review points discussed or first reported in relatively inaccessible journals. In fact, one of the merits of this book is the restriction of the citations to fewer and more fundamental papers. The discussions of most of the points reflect the broad experience of the author in the various fields of plant physiology which depend upon chemical facts and theories.

Comparison of this translation with the original German edition, however, will show many radical changes in the subject matter. These are due to the revisions which Dr. Kostychev made late in 1929 in order to bring this edition as nearly up-to-date as possible. Several fields of biochemistry and physiology developed so rapidly between 1926 and 1930 that important modifications in the text were necessary. The most important changes were made in connection with plant enzymes, photosynthesis, denitrification, vitamins, cell-free fermentation, and the theories of fermentation and respiration for which new and important evidence has been obtained. In addition to less extensive revisions at other points, there are many new citations included and some minor errors corrected. These changes and additions by the author have been incorporated as though they had been a part of the original text.

To the original forty-four text figures has been added one of the Davis-Hoagland apparatus (Fig. 32) for controlling the environmental conditions under which plants can be grown in studies of their mineral nutrition and

similar problems. I am indebted to Dr. A. R. Davis and the American Society of Plant Physiologists for the use of this cut.

In connection with the names of Russian scientists and journals which appear frequently in this book, it has seemed best to avoid the danger of errors in transliteration into English equivalents by taking the spelling as used in the German text. The one exception is that of the name of the author of this book wherever it occurs in the text, the form Kostytschew being kept in the citations. In the case of Russian monographs, the titles have been translated from German to English. The names of authors and journals or books as used in others citations have been left in the form used by the author.

The form of citation is a condensation of that in the German edition. It uses more or less standardized abbreviations for journals, with the series number or letter in parentheses, a boldface Arabic numeral (followed by a colon) for the volume number, and ordinary Arabic numerals (followed by a period) for page and year numbers. The authors' names are given in bold-face type in the citation in order to facilitate reference to original papers. **Idem** denotes a repetition of the preceding author or authors while *Ibid.* indicates a repetition of the preceding journal or monograph.

The index has been enlarged by the insertion of more detailed subject entries and the names of plants mentioned, and by the compilation of an index to the authors which is merged with the subject index. Authors' names are placed in bold-face type as are the pages on which such names appear in a complete citation.

CHARLES J. LYON.

DARTMOUTH COLLEGE

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INTRODUCTION

Physiology is a science the object of which is to investigate the vital phenomena of organisms. In its fundamental character, physiology is a unified science because the principal vital processes of all organisms are absolutely constant in their nature. The separation of physiology into two branches—physiology of plants and physiology of animals—does not prevent us from considering a whole series of fundamental vital phenomena for processes which are equally necessary for plants and animals.

Between the typical representatives of the animal and plant worlds, however, we can recognize some important biochemical contrasts. Animals feed chiefly on complex organic substances which they break up into more simple chemical compounds; on the contrary, plants are nourished chiefly by the simplest inorganic substances which they assimilate by synthetic processes. It is evident that this distinction is only a generalization for various syntheses occur in animal cells and there are numerous cases of cleavage of organic substances within plant cells. This antithesis between the plant and animal worlds refers to the resultant of the total material transformations which take place in relation to the various biological processes.

The body of the higher animals is more complex in its organization than that of the higher plants. Through this greater complexity, a more complete division of labor is attained in animals. Consequently it is more convenient to study the functions of single organs and tissues in the animals while in plants it is easier to observe the general vital phenomena, because in plants the fundamental processes take a much simpler form than is the case in the organs of animals.

Long ago Descartes referred to the analogy between organisms on the one hand and the various machines or mechanisms on the other. A living organism is actually constructed for a purpose; in other words, the structure of living things is based, so to speak, on a plan according to which the functions of the organism concerned condition its bodily structure (Cuvier). Of course, the structure of the simplest organism is much more complex than that of machines or of a factory, which is a system of machines. A living organism, like a factory, performs certain chemical and mechanical operations but in addition it has the power to develop, to renew its tissues and organs and, what is particularly remarkable, to reproduce or, in other words, to beget organisms like itself. No complex factory has any such capacities.

Nevertheless, the analogy between organisms and chemical factories is far-reaching. It will help us to define the general problems of plant physiology. Physiology endeavors to trace the phenomena of life to the general laws of chemistry and physics, *i.e.*, in the final analysis to state all the chemical processes in organisms by chemical equations and to interpret the energy transformations

and the entire structural features on the basis of physical laws. It need hardly be mentioned that this goal is not yet reached, but it must be emphasized that the chemical physiology of plants has already made great progress and developed rapidly, especially during the past decade.

On the basis of the present knowledge, we divide plant physiology into two large sections—chemical and physical plant physiology. The chemical physiology deals with the questions of nutrition in the broader sense of the word, *i.e.*, with the assimilation of various nutrient substances and with the handling of them within the plant body. The physical physiology seeks to explain the mechanisms of the following processes:

1. Absorption and movement of substances.
2. Growth and internal structure.
3. Movements of plants.

In some text books of plant physiology, the first section of physical physiology is merged with the entire chemical physiology into a homogenous section on "plant nutrition." This method is not used in the present book for the following reason. The biochemical studies are sharply distinguished from the investigations of the laws of physical physiology by reason of the methods used, these being of predominant significance in all experimental science; hence this book is written on methodological foundations.

Chemical plant physiology is often called "biochemistry of plants," because in its present state it is actually nothing but a special section of general chemistry. It deals only with questions of material transformations in plants which are regarded merely as the place where these changes take place. The structure of the plant body is given little consideration in chemical plant physiology because it has little bearing on the actual questions of chemical physiology. The current chemical physiology is not yet equal to the questions of energy yield and energy changes involved in the physiological changes of substances. However, it must be borne in mind that physiology in the true sense of the word must regard the properties of the organism as a complex apparatus. Likewise physical plant physiology studies the machine-like structure of plants but it has lost the connection with pure physics in the study of many basic questions and so it does not fully penetrate the problems of true physiology. Physical physiology is most deficient in that it traces complicated life processes to simple processes which of themselves cannot be explained thoroughly by the general laws of physics.

In spite of all this, the scientific work in both branches of plant physiology is fruitful and most promising. For example, if it is eventually possible to investigate the characteristics of the plant as a complex apparatus, such studies will rest on a firm chemical foundation. In the same way plant respiration, the source of energy in plants, is now being studied only chemically. For the present we do not know how the chemical energy of the combustion of carbohydrates is used in the processes of growth and differentiation of tissues. This transformation of energy can be investigated only after the chemical side of the respiratory process has been made clear. In like manner, at least one field

of work depends on physical physiology for the necessary preparation. If it is finally possible to analyze the simplest type of movement in response to a stimulus on the basis of laws of mechanics and physics, this analysis will have a general significance only if all the complex types of plant movement are already known as combinations of the simplest movements.

Chemical plant physiology has been worked out much more completely than physical physiology. This is so because chemical plant physiology is of far-reaching significance for agriculture, medicine and several technical trades. Consequently a whole army of agricultural chemists, pharmacists, brewers, etc. have coöperated in this branch of physiology. The greater part of the achievements of chemical plant physiology is not due to the work of professional botanists but to that of the technical workers just mentioned, especially the early work.

This volume deals with the chemical physiology. First there is an exposition of the assimilation of the elements required by plants. Then follows the description of the transformations of the assimilated foods in connection with the various vital requirements of plants. Finally there is a discussion of the biochemical processes which furnish energy to plants. As it has already been indicated, all these processes are treated as chemical reactions. An exhaustive discussion of the physiological significance of specific cell and tissue structure is entirely lacking; all the plants are considered as a single medium. According to the view of the author, it is possible only by this method to give a connected description of the biochemical processes in plants. This method of presentation makes the book accessible to the pure chemists, which is important in so far as the more recent achievements of chemical plant physiology have also acquired a great importance for the various branches of general chemistry. On the other hand, a schematic arrangement of plant structure can be worked out without difficulty by considering the purely chemical side of the life processes. Indeed, knowledge of plant anatomy is essential for an understanding of the coördination of the various biochemical processes. As for the chemical nature of the physiological processes, there is no evidence that this could be influenced in any way by the structural adaptations.¹ This assumption was most improbable to begin with.

In a second book [as yet unpublished] the organization of the plant body and the structure of individual plant tissues will be brought to the foreground in connection with the questions to be discussed. Physical plant physiology falls into three sections: 1. Absorption and movement of substances: 2. Growth, correlations and tissue differentiations: 3. Plant movements. Since the questions of heredity and variability form a special branch of science, they will be considered only in so far as it is essential for the correct understanding of related problems.

¹ Of course, boundary surface phenomena are processes which depend on phases (p. 2) but not on definite structures.

CHEMICAL PLANT PHYSIOLOGY

CHAPTER I

THE FOUNDATIONS OF CHEMICAL PLANT PHYSIOLOGY

I. THE PROBLEMS AND PRESENT STATUS OF CHEMICAL PLANT PHYSIOLOGY

As already mentioned in the introduction, chemical plant physiology now endeavors to trace all physiological transformations of substances to general, known chemical reactions. Only the attainment of this goal will make it possible to investigate the physiological significance of these transformations. For a long time it was impossible to state this problem in such simplified form. It is only recently that circumstances have allowed a direct attack on the pertinent questions. Previously it seemed as though the most important material transformations in organisms were not in accord with the fundamental laws of chemistry and physics. For example, the important question of the soil nutrition of plants came up against the following apparently paradoxical facts: various salts are not soluble in the soil solution and in the cell sap of plants in the proportions to be expected on the basis of theoretical chemistry. Exactly the same thing can be found in the biochemistry of animals; certain salts are dissolved in milk and in the blood while the same salts are scarcely soluble in water, although upon precipitation of the proteins of these liquids these soluble salts are partially carried down. Another apparently paradoxical fact is seen when, in the respiration of animals or plants, carbon dioxide and water are formed by the oxidation of substances which burn only at very high temperatures outside the organism. Likewise the synthesis of substances of high molecular weight is brought about in organisms under conditions which seem to be theoretically inconceivable from the chemical standpoint.

On account of a large number of analogous difficulties, the so-called "chemical plant physiology" did not justify its name up to the opening of the present century. There was no chance for a chemical analysis of the nature of the biochemical processes, so the experimental study had to deal with secondary problems and particularly with the influence of various external factors on the biochemical processes. If we open any manual of plant physiology written at the end of the 19th or the opening of the 20th century we find the so-called "chemical part" of the book filled with descriptions of the effects of various, often secondary, external factors upon physiological functions. As regards the actual chemistry of the material transformations which alone should form the true content of chemical physiology, we find at most only some incidental observations. This abnormal condition, however, awaited the development

of some special sections of physical chemistry, a natural offshoot of physiology, which again meant the investigation of a whole series of facts which at first seemed unattainable. Moreover, the biochemists were giving their attention to the complex chemical reactions in order to establish intermediate stages by which the chemical nature of these processes becomes conceivable.

To be sure, this work is only just begun. However, it is important that this problem can be solved experimentally; success is only a question of time. The ever growing interest in the physiological chemistry of plants, which in recent years has continued to work out biochemical questions, is due to the practicality of altering the nature of the working methods in the field and finally of confining the attention to the questions which form the core of chemical plant physiology.

2. HETEROGENEOUS SYSTEMS AND BOUNDARY SURFACE PHENOMENA

In chemistry a homogeneous system refers to a mixture of parts physically alike, such as an aqueous solution of various substances or a mixture of gases.

On the contrary we designate as "heterogeneous" a system which consists of various parts separated from each other by boundary surfaces. The single parts of the system are called "phases." Thus the aqueous solution of different substances forms a heterogeneous system with the air with which it is in contact; here the system consists of a liquid phase and a gaseous phase. The characteristics of heterogeneous systems are manifested chiefly at the boundary surfaces which separate the phases from each other and at which we find the specific phenomena called "boundary surface phenomena." The greater the development of surfaces in a heterogeneous system, the greater the boundary surface activity.

In such a system, each boundary surface is the seat of a specific force—surface tension—which is described in elementary text books of physics. The magnitude of the surface tension can be stated on the basis of the rise of liquids in capillary tubes: $\gamma = \frac{r \cdot h \cdot s}{2}$, where r is the radius of the capillary tube, h is the height of rise and s is the specific gravity of the liquid. The physical chemistry of boundary surface phenomena is also known as capillary chemistry.

A simpler method is now preferred for the determination of surface tension; a count is made of the number of drops in a known volume of liquid, since the volume of each drop is directly proportional to the surface tension and to the diameter of the capillary tube. A simple apparatus called the *stalagmometer* is used for these determinations. It consists of a calibrated, bent capillary tube with a polished dropping surface. The volume of the single drops can also be noted after each has left the calibrated capillary tube. This apparatus which is analogous to the stalagmometer, is called a *viscostagometer*. If the surface tension of water is taken as 100, the surface tension of another liquid can be calculated by the formula $\gamma_1 = \frac{ZS_1}{Z_1}$, where Z and Z_1 are the number of drops in equal volumes of water and the second liquid, and S_1 is the specific gravity of the liquid.

The newest method of measuring surface tension is to use a torsion balance to determine the force required to pull a small platinum ring of a certain diameter

from the surface of the liquid to be studied.¹ The surface tension is calculated from the following formula:

$$\alpha = p - r\sqrt{2psg} + \frac{r+3}{4}r^2sg.$$

In this case α is the surface tension in dynes per square centimeter, p is the maximum pull on the ring in dynes divided by $4\pi R$, r is the radius of the cross section of the wire from which the ring is made, and sg the density of the liquid times the gravitational constant.

The Gibbs-Thomson theorem² is important for physiology. It says that the dissolved substances which lower the surface tension of the solvent, collect at the surface. This increase in concentration is called adsorption. Accordingly, all substances which lower the surface tension are *positively adsorbed* while those which raise surface tension are *negatively adsorbed*. If a solution of amyl alcohol in water is thoroughly shaken it forms a rich froth. If this froth is removed and its alcoholic content determined, it will be found that the concentration of alcohol is greater in the froth than in the original solution.³ Amyl alcohol actually increases the surface tension of water. A solution of a protein in water gives a permanent froth in which the surface increases and the foam hardens to a solid phase upon long standing. In this case the concentration of protein at the surface becomes so high that the protein is thrown out of solution.⁴ Protein lowers the surface tension of water.

Adsorption is a reversible process. The amount of substance adsorbed at the surface of the solution is proportional to the amount in solution. This equilibrium can be regarded as similar to the chemical equilibrium of reversible reactions, *i.e.*, it can be assumed that it occurs when in a unit of time the amount of substance passing into the surface is equal to that diffusing from the surface into the liquid. In order to ascertain the ratio of the adsorbed to the dissolved substance at various concentrations of the latter, the work must be done at a constant temperature because fluctuations in temperature have a marked effect on adsorption. Figure 1 is the so-called adsorption isotherm.

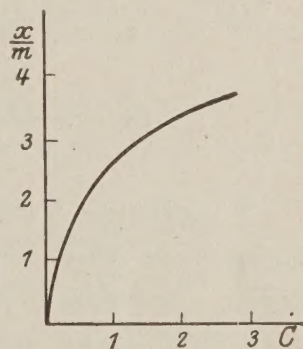


FIG. 1.—Adsorption isotherm. (After Freundlich.)

From the curve it is apparent that the adsorption at low concentrations is relatively greater than at high concentrations.⁵ After reaching a certain concentration the increase in adsorption practically ceases: this is known as saturation of the surface.

¹ Brinkman, R. Abderhalden's Handbuch d. biol. Arbeitsmethoden, Abt. IV, Teil 4, page 1417 (1927).

² Gibbs, J. W. Thermodynamische Studien, p. 258. 1892; Thomson, J. J. Application of dynamics to physics and chemistry, pp. 191 and 251.

³ Benson. Amer. Jour. Phys. Chem. 7: 532. 1903; Donnan and Barker. Proc. Roy. Soc. London (A) 85: 557. 1911.

⁴ Ramsden. Zeitschr. f. physikal. Chem. 47: 343. 1904; Metcalf. Ibid. 52: 1. 1905; and many others.

⁵ Freundlich. Capillarchemie. 1909; Idem. Zeitschr. f. physikal. Chem. 57: 385. 1907; and others.

The dependence of adsorption on the concentration of the substance to be adsorbed is expressed by the following empirical formula:

$$\frac{x}{m} = \alpha c^{\frac{1}{n}}$$

Here $\frac{x}{m}$ is the intensity of adsorption *i.e.* the amount of adsorbed substance calculated per unit of boundary surface of the adsorbent, α is a constant characteristic of the substance which is being studied, c is the concentration of substance in solution while $\frac{1}{n}$ is another constant.¹ If the logarithms be taken of both sides of this equation, the result is the equation of a straight line:

$$\log \frac{x}{m} = \frac{1}{n} \log c + \log \alpha.$$

If the logarithms of the variable c be plotted as abscissae and those of the variable $\frac{x}{m}$ as ordinates, the graph is a straight line (Fig. 2) if the process under consideration is actually adsorption. This is a useful test in experimental procedure.

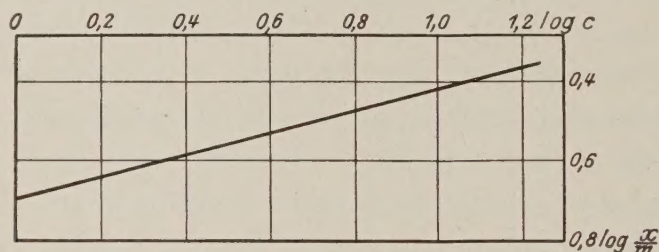


FIG. 2.—Logarithmic graph of adsorption isotherm. (After Morawitz.)

Experience teaches that different substances are not equally adsorbed. Substances which are strongly adsorbed are called surface active while those which are weakly adsorbed are said to be surface inactive. The boundary surface phenomena are relatively weak at the boundary between a liquid and the air since this surface is weakly developed if it forms no froth. On the contrary, these phenomena are often much stronger at the junction of a liquid and a solid phase, especially if the latter is finely divided throughout the liquid. Two immiscible liquids can form a fine emulsion with an enormous development of boundary surfaces. It has been known that finely powdered, strongly porous bodies such as charcoal adsorb various substances when shaken in aqueous solution. Charcoal is also a strong adsorbent of gases. The importance of the adsorbent is not determined by the surface development alone but its specific nature apparently plays a rôle which is not yet clearly understood.

It is very important that different substances displace each other from surfaces according to the relative amounts and the surface activities of the substances concerned.² The adsorption of electrolytes is due to the adsorption of

¹ α is the intensity of adsorption of a given substance when $c = 1$, *i.e.*, the amount which is adsorbed by a unit of the other phase at a concentration of 1, $\frac{1}{n}$ is the coefficient of curvature of the adsorption isotherm. It is usually not greater than 0.7 and not less than 0.1.

² Michealis und Rona. *Biochem. Zeitschr.* 15: 196. 1908; Rona und v. Toth. *Ibid.* 64: 288. 1914; Freundlich und Poser. *Kolloid-chem. Beihefte.* 6: 297. 1914.

single ions; the latter are all adsorbed to the same degree and it has been found that the following series hold:

For anions: $\text{SO}_4 < \text{HPO}_4, \text{Cl} < \text{Br} < \text{NO}_3 < \text{I} < \text{CSN} < \text{OH}$

For cations: $\text{Na}, \text{K}, \text{Rb}, \text{Cs}, \text{NH}_4 < \text{Ca}, \text{Mg} < \text{Zn} < \text{Al} < \text{Hg}, \text{Ag}, \text{H}.$

Hence the adsorption of an electrolyte resolves itself into the additive adsorption of the cations and anions, but on account of the strong electrical charges of ions their mutual power of attraction is so great¹ that it is impossible to separate two oppositely charged ions from each other. On account of the strong electrostatic attraction, a strongly adsorbed cation draws with it a weakly adsorbed anion and *vice versa*. In case the adsorbent can itself split off soluble ions, the resultant adsorption becomes an exchange of ions.

Colloids can lower the surface tension markedly. Later it will be shown that colloids are divided into two groups—hydrophobe (suspensoid) and hydrophile (emulsoid) colloids. The first are characteristically insoluble; their solution produces a heterogeneous system which makes them surface active; on the contrary, hydrophile colloids are soluble and are often adsorbed so strongly that they form a gelatinous film at the surface of the liquid or at the junction of two immiscible liquids. This may possibly be the basis of the property of every protoplast to form a surface film when the cell membrane is removed or after continued plasmolysis.

The so-called electro-kinetic phenomena are closely bound with the matter of adsorption. They consist of the following.

If an electric current be passed through water in which are suspended many fine solid particles, all these particles migrate to one pole and usually to the anode. This is the so-called *cataphoresis*.² On the contrary, if a solid phase be fixed (by migrations to a porous clay vessel or suitable porous body) the water is set in motion in the potential gradient; it flows toward the opposite pole which is usually the cathode. This phenomenon is known as “electro-osmosis”;³ it probably plays an important physiological rôle. Cataphoresis and electro-osmosis show that the solid and liquid phases are oppositely charged.

The following equation applies to the rate of cataphoresis:

$$V = \frac{\varphi \cdot E \cdot D}{4\pi\eta},$$

in which φ = potential difference at the junction of the solid and liquid phases,

E = fall of potential in the current,

D = dielectric constant of the liquid,

η = internal friction constant.

It is worthy of note that the rate of cataphoresis does not depend on the size of the particles.

The charge of the solid particles and of the water may be due to two reasons. In the first place, substances with a higher dielectric constant are usually positively charged in relation to substances with a lower dielectric constant.⁴ Water has a very high dielectric constant so that most other substances are charged negative to water. A second, more important reason is that one is practically never dealing with absolutely pure water; a certain amount of elec-

¹ A gram equivalent of ions bears a charge of 95,540 coulombs.

² Wiedemann, G. Pogg. Ann. 87: 321. 1852; Quincke. Ibid. 113: 593. 1861.

³ Perrin. Compt. rend. 136: 1388. 1903; 137: 513. 1903; Idem. Jour. chim. phys. 2: 601. 1904; 3: 50. 1905.

⁴ Coehn, A. Wiedemanns Ann. 64: 217. 1898; Coehn und Raydt. Ann. d. physik. 30: 777. 1909.

trolytes is always dissolved in it. Even minute traces of these substances exert a large effect upon electro-osmosis as well as upon cataphoresis.¹ Electrolytes with a certain charge can even produce a reversal of the movement so that the solid particles go to the cathode and water to the anode, due to the discharge of these particles. In this way the charge of the solid particles and of the water is conditioned by ions. An explanation of the effects of electrolytes on electro-kinetic phenomena, at first sight paradoxical, is as follows.²

As already explained, the adsorption of electrolytes is an addition phenomenon because it consists in the adsorption of anions and cations. Usually one ion is more strongly adsorbed than the other. This difference cannot be determined quantitatively by chemical analysis because the adsorption follows electrical equivalents and the more strongly adsorbed ion, as the result of the electrostatic force of attraction, pulls the other ion to the adsorbent. Consequently the difference between the amounts of adsorbed anions and cations is so small that it can be determined only by extremely sensitive electrometric methods, since the ion that is held in excess confers its charge on the adsorbent. The detection of this charge is possible only because the electrical charges of ions are especially strong.³ The potential difference between solid particles and water usually amounts to only a few hundredths of a volt.

There is another possible origin of the charge on solid particles. Many substances such as weak bases or acids and, since there are no absolutely insoluble substances, solid particles suspended in water, give off H or OH ions into solution. The second ion, which is complex or colloidal in nature, remains adsorbed to the particle.

In conclusion it should be mentioned that adsorption in its pure form is a physical phenomenon. We have already (page 3) seen that there can be an increase in the amount of adsorbed substance only by raising the concentration of the substance held in solution; however, in the case of a chemical reaction in a heterogeneous system other factors are to be noted. Thus there is an increase in the amount of CO₂ passing over into CaCO₃ during the fixation of CO₂ by solid calcium oxide at a constant concentration of CO₂.⁴ On the other hand, there is often no sharp distinction between adsorption and chemical union since both phenomena are based on electrical forces.

3. COLLOIDS IN GENERAL

The greater part of physiological transformation is done in so-called colloidal media. Graham,⁵ the founder of colloidal chemistry, made a sharp distinction between "crystalloids" *i.e.*, substances of crystalline structure and which diffuse easily through membranes (such as animal bladders, parchment paper, etc.), and colloids—amorphous substances which diffuse slowly. Now we speak of a "crystalloid" and a "colloidal" form of every substance, because the same substance may be found in either form according to the solvent, the concentration and other conditions.⁶ However, since many substances of high molecular weight are almost always found in the colloidal state it is practically correct to call them "colloids."

From the modern standpoint there is no doubt but that the particles in a colloidal solution are larger than those in a true solution. Substances of high

¹ Perrin. *loc. cit.*; cf. also Languier des Bancelles. *Compt. rend.* 149: 316. 1909; Barrat und Harris. *Zeitschr. f. Elektrochem.* 18: 221. 1912; Gyemant. *Kolloidzeitschr.* 28: 103. 1921.

² Freundlich. *Zeitschr. f. physikal. Chem.* 73: 385. 1910; Freundlich und Schucht. *Ibid.* 85: 641. 1913; Donnan and Ridsdale Ellis. *Ibid.* 80: 597. 1912; Kruyt. *Kolloidzeitschr.* 22: 81. 1918.

³ The experimental proofs of the accuracy of this view of the effect of electrolytes on cataphoresis are so numerous that it is impossible to give them here. The details can be found in text books of physical chemistry.

⁴ Le Chatelier. *Compt. rend.* 102: 1243. 1886.

⁵ Graham. *Liebigs Ann. d. Chem.* 121: 1. 1861.

⁶ Weimarn, P. *Zeitschr. f. Kolloidchem.* 2: 76. 1907; 8: 24. 1911; Idem. *Zeitschr. f. physikal. Chem.* 76: 212. 1911; Ostwald, Wo. *Kolloidchem. Beihefte.* 4: 1. 1912.

molecular weight tend to form molecular complexes, so that a solution includes single molecules of the substance but within the solution there are also molecular aggregates which, under certain conditions, become so large that they can be detected directly by any one of various devices. The extreme case occurs when all the particles in the solution can be recognized as being of a different structure than the surrounding medium (heterogeneous system), while the characteristic feature of a true solution is the impossibility of finding physical inequalities throughout the liquid. If all the particles (disperse phase) suspended in the liquid are visible with the highest magnifications of the microscope, the diameter of a single particle is not less than $150\mu\mu$.¹ In this case there can be no question of a solution but we are here dealing with a suspension in water. If the particles in suspension are not visible in the ordinary microscope they may often be detected by the ultramicroscope. Such particles are said to be submicrons; particles as small as $4\mu\mu$ can be seen with the ultramicroscope.

The principle of the ultramicroscope² is based on the so-called "Tyndall effect."^a If the liquid to be examined is placed in a transparent glass vessel and observed from above through a microscope while a horizontal beam of light is passed through from the side, the light will be dispersed by the particles in suspension. From each particle^b a partly polarized cone of light is sent up into the microscope. In this way we observe in the microscope bright little diffraction discs (sections of cones), the diameters of which are considerably larger than those of the particles which disperse the light. Hence it is evident that the ultramicroscope in its present form gives no true idea of the form of the particles under observation. The liquid in which the ultramicroscope shows no lighted particles is taken to be optically void. True solutions of substances of other than high molecular weight are optically clear as a rule, but it should be noted that compounds of high molecular weight, such as cane sugar or raffinose, show the Tyndall effect to a slight degree.³

By a colloidal solution we mean a "micro-heterogeneous system," which consists of a liquid phase and either a stable suspension of very small particles, or tiny drops of a liquid. The latter is immiscible in the continuous phase.

In the first case we are dealing with "suspensoids" or hydrophobe colloids; in the second case, with "emulsoids" or hydrophile colloids. The most important differences between these two categories of colloidal states are as follows.

¹ Micron (0.001 mm.) is written as μ and millimicron or 0.001 micron (*i.e.*, a millionth of a millimeter) is written $\mu\mu$. [It has been suggested recently (Uhler. *Science*. 65: 232-233. 1927; 70: 606-608. 1929) that $m\mu$ be used in place of $\mu\mu$ which was erroneously introduced for 10^{-9} meter.]

² Siedentopf und Zsigmondy. *Drud. Ann.* 10: 1. 1903; Zsigmondy. *Zur Erkenntnis der Kolloide*. 1905; Cotton et Mouton. *Compt. rend.* 136: 1657. 1903; [Freundlich. *Colloidal and Capillary Chemistry* (trans. by Hatfield). Pp. 387-390; Zsigmondy. *Colloids and the Ultramicroscope*; Gortner. *Outlines of Biochemistry*. P. 80 ff. The use of ultraviolet light with special condensers and objective lenses gives better resolution but visual observations must be replaced by photographs. Cf. Barnard. *Jour. Roy. Micros. Soc.* 49: 91. 1929. It should also be noted that dark-field condensers are commonly used in place of the more elaborate ultramicroscope.]

^a This was described by Tyndall in *Phil. Mag.* (4) 37: 384. 1869 though earlier workers including Faraday probably noticed it.—*Ed.*

^b Such particles will not be stationary but will oscillate rapidly and thus exhibit the Brownian movement which is a characteristic of colloidal solutions. This movement is described in detail by Gortner *loc. cit.* P. 55 ff.—*Ed.*

³ Lobry de Bruyn et Wolff. *Recueil des travaux Chim. des Pays-Bas.* 19: 251. 1900; 23: 155. 1904.

If a solution of a hydrophobe colloid is evaporated to dryness, the residue will not go into solution a second time when more solvent is added; on the contrary, the residue from a hydrophile colloid can be easily brought into solution again if it is still chemically unchanged. This depends on the fact that hydrophobe colloids have no affinity for the solvent. Hydrophile colloids, on the contrary, attract water; hence, a liquid disperse phase will always result when the solvent is added. By this expression we understand the condition of a fine division of one substance in another. For hydrophobe colloids, which include chiefly inorganic gels such as $\text{Fe}(\text{OH})_3$ and sulphides, the degree of dispersion is not that of a molecular solution; in the case of the hydrophile colloids, to which proteins, carbohydrates of high molecular weight, lecithin and other physiologically important substances belong, the degree of dispersion often approaches the condition of a true (molecular) solution,¹ on the basis of the osmotic pressure. However, under the influence of various factors the hydrophile colloids acquire the properties of the hydrophobes.

Another important distinction between the hydrophobe and hydrophile colloids is that solutions of the latter exhibit an appreciable internal resistance or viscosity and the surface tension is considerably less than that of the pure solvent. On the contrary, the viscosity and surface tension of solutions of hydrophobe colloids are almost never different from those of the solvent.² These properties are explained by the present conception of colloids. It has already been mentioned that the surface tension is often lowered by the solute but the hydrophobe colloids do not act as molecules in solution; hence, they cannot affect the surface tension. As for the internal friction, it is that of the tiny particles of the dissolved substances against the particles of the solvent. The hydrophobe colloids do not go into true solution and cannot increase the internal friction. Their particles are sharply separated from the surrounding liquid phase. On the other hand, the hydrophile colloids are liquid and their particles attract a large number of water particles so that the disperse phase contains a large percentage of the total volume of the liquid, e.g., 88% in a 9.5% solution of casein.³ It may be supposed that the greatly swollen colloidal particles push by each other and are thereby deformed, so to overcome the internal friction in these cases the elastic forces must be taken into account.⁴ On the basis of all these facts, it may be assumed that the "solution" of a hydrophobe colloid is similar to a suspension (finely divided solid particles in a liquid) while that of a hydrophile colloid is an emulsion (a mixture of two liquids).

4. PROPERTIES OF HYDROPHOBE COLLOIDS

It has already been mentioned that this category includes substances of little importance in physiology, such as the oxides and sulphide of a metal and

¹ Hufner und Gansser. Arch. f. Physiol. for 1907. P. 209; Blitz, W. Zeitschr. f. physikal. Chem. 91: 705. 1916; Sørensen. Ibid. 106: 1. 1919.

² Linder and Picton. Jour. Chem. Soc. (London) 67: 63. 1895; Zlobicki. Bull. acad. sci. Cracovie for 1906. P. 488; Friedländer. Zeitschr. f. physikal. Chem. 38: 385. 1901; Woudstra. Ibid. 63: 619. 1908.

³ Hatschek. Kolloid-Zeitschr. 12: 238. 1913.

⁴ Hatschek. Kolloid-Zeitschr. 8: 34. 1911; Hess, W. R. Ibid. 27: 154. 1920; Rothlin. Biochem. Zeitschr. 98: 34. 1919; Loeb, J. Jour. Gen. Physiol. Vols. 3 and 4. 1920-21.

pure metals in a finely divided state. Yet in these colloids the colloidal properties are especially well marked and under certain conditions the hydrophile colloids behave in the same manner; hence the hydrophobe colloids must be described.

Hydrophobe colloids show the Tyndall effect clearly, deviate from the usual laws of osmosis¹ and do not diffuse through membranes, such as collodion, parchment, animal bladders, etc., through which electrolytes pass easily. Of particular interest are the cases in which the colloid is ionized because one ion passes into solution while the other remains in the colloidal state. The surprising osmotic phenomena which then appear are often described under the name of "Donnan equilibrium."

Upon the difficulty of filtration of colloids depends the so-called "ultra-filtration" which makes it possible to separate the dissolved colloid from the disperse medium. For this purpose an ultrafilter is prepared by moistening filter paper with collodion, gelatine or a similar substance² and hardening it with water. In this way it is possible to classify colloids according to the size of their particles since the filter can be prepared with pores of any size and the colloidal mixture can be resolved into its component parts. Of course the capacity of colloids to pass through the ultrafilter depends not only upon the size of the particles but also upon adsorption phenomena and swelling. The latter will be described later.

As a result of the wide dispersion of a colloid, its solution forms a heterogeneous system with large boundary surfaces, and adsorption phenomena become very noticeable. The unequal adsorption of single ions by the colloidal particles produces an electrical charge on the latter,³ and colloidal solutions show cataphoresis⁴ when an electric current is passed through them. Colloidal substances are very difficult to prepare in pure form because when the colloid is thrown out of solution by flocculation a large amount of the adsorbed electrolytes and other components of the mixture is carried along.

The most useful method of separating colloids from the adsorbed materials is "dialysis." The colloidal solution is placed in a sack of parchment paper, collodion or animal bladder (Fig. 36, page 287), the area of the walls being made as great as possible and the sack not quite filled with the solution, since the level of the liquid is usually raised at the close of the dialysis. The sack is then suspended in a large vessel and exposed to a current of water. Of course, the upper part of the sack must be kept above the level of the water in the vessel. The substances which diffuse through the membrane are washed away and their concentration in the solution gradually falls. We know that adsorption is a state of dynamic equilibrium and depends entirely on the concentration of the given substance in the liquid phase. If this concentration is almost zero, adsorp-

¹ Duclaux. *Compt. rend.* 140: 1544. 1905.

² Bechhold. *Zeitschr. f. physikal. Chem.* 60: 257. 1907; 64: 328. 1908; Zsigmondy. *Zeitschr. f. angewandte Chem.* 26: 447. 1913; Zsigmondy und Bachmann. *Zeitschr. f. anorg. Chem.* 103: 119. 1918; Ostwald, Wo. *Kolloid-Zeitschr.* 22: 72 and 143. 1918.

³ Linder and Picton. *Jour. Chem. Soc. (London)* 67: 63. 1905; see also Barus und Schneider. *Zeitschr. f. physikal. Chem.* 8: 278. 1891.

⁴ Whitney and Blake. *Jour. Amer. Chem. Soc.* 26: 1339. 1904; and many others.

tion also reaches a minimum value. After several days of dialysis in a stream of running water, it is well to finish the dialysis in distilled water; even then a small amount of the impurity always remains adsorbed.

If a certain amount of electrolyte be added to a solution of a hydrophobe colloid, the charge on the colloidal particles is lessened¹ (as we have already seen in the consideration of the adsorption of electrolytes by particles suspended in a liquid). When completely discharged or nearly at this "isoelectric" point, the solution becomes unstable and the colloid is precipitated.² This important fact has a great physiological significance. It is quite conceivable that the coördination of biochemical processes in the protoplasm, and hence the entire regulation of vital processes, is effected by means of changes of the medium in which all these processes take place, because the protoplasmic colloids are able to change their properties and, under certain conditions, to behave like typical hydrophobe colloids. Hence it is very interesting to consider the effect of electrolytes on colloids in some detail.

The first extremely important principle is that the flocculation of negatively charged colloids is brought about by cations,³ since cations neutralize the electrical charge of these colloids. The second rule states that the power of precipitation of an ion is a function of its valence.⁴ This is shown by the following table.

Precipitation of equal amounts of the negatively charged colloid As_2S_3 is brought about by the following amounts of electrolyte (in 0.001 mol. per liter).

Monovalent metals	Divalent metals	Trivalent metals
NaCl..... 51.0	MgCl ₂ 0.717	AlCl ₃ 0.093
KNO ₃ 50.0	CaCl ₂ 0.649	Al(NO ₃) ₃ 0.095
KCl..... 49.5	SrCl ₂ 0.635	ICl ₃ 0.073
LiCl..... 58.4	Ba(NO ₃) ₂ 0.687	$\frac{1}{2}\text{Ce}_2(\text{SO}_4)_3$ 0.092
NH ₄ Cl..... 42.3	ZnCl ₂ 0.685	

Both these principles are easily explained if it be assumed that the effect of the electrolyte is due to its adsorption. Since only single ions are adsorbed a colloid must, of course, adsorb an excess of ions with the opposite charge and so neutralize its own charge. But we know that the unequal adsorption of single ions cannot go far because the oppositely charged ions follow the others to the surface. Since a negatively charged colloid can adsorb only a small excess of cations, the valence of each cation becomes significant. It must be borne in mind that we are dealing with the effects of ions on colloids with a very low potential. Aside from these theoretical considerations there also exist direct

¹ Of course, a colloid adsorbs the oppositely charged ions with greater intensity.

² Hardy. Jour. of Physiol. 24: 301. 1899; Zeitschr. f. physikal. Chem. 33: 385. 1900.

³ Hardy. *loc. cit.*; Jour. of Physiol. 33: 251. 1905.

⁴ Freundlich. Zeitschr. f. physikal. Chem. 44: 135. 1903; 73: 385. 1910; Freundlich und Schucht. *Ibid.* 80: 564. 1912; Mines. Jour. of Physiol. 42: 309. 1911.

experimental results in favor of the relationship between the adsorption of ions and the effect of electrolytes.¹

Finally, there is the theoretical question as to why the colloid is precipitated when its charge is gone. At present the question can be answered only hypothetically and the most probable explanation is as follows.² Between the fine particles there is an attraction which, combined with the Brownian movement that constantly brings the particles into the sphere of attraction, tends to bring about the flocculation. That this actually does not occur is explained by an electrical repulsion of the similarly charged colloidal particles which counteracts the force of attraction. So the weaker the charge the less stable is a colloidal solution.

It is worthy of note that in the case of an excess of electrolytes or of an effect of another salt on the precipitated colloid, the latter again passes into solution but its properties are thereby changed. This process is called peptization and it seems to have an important physiological significance. It is due to a recharging of the colloid. This repeated precipitation and subsequent peptization of a colloid is possible because first one and then another ion is adsorbed in excess.³ In a similar way there is a precipitation when two oppositely charged colloids come together.⁴ In this case a subsequent peptization is possible through the loss of charge on the disperse phase.⁵ That is, the particles of a colloid are enveloped by another colloid; this process can be observed directly with the ultramicroscope and colloids of different colors.⁶ It is very remarkable that the precipitation of one colloid by another is practically complete if large quantities of the precipitant are added rapidly. If the precipitant is added gradually the colloid in solution appears to adapt itself and is not precipitated by the other. There is no good explanation of this type of behaviour. It should be compared with the so-called "agglutination" or separation of the phases of true suspensions (finely divided precipitates suspended in a liquid). Here the particles which migrate to the anode in cataphoresis are precipitated by positively charged particles and vice versa. Of both theoretical and practical interest is the process of agglutination of bacteria from liquid media. The bacteria are fixed by a specific colloid called agglutinin. Actually there are two stages to the process. In the first, the bacteria which consist chiefly of hydrophile colloids (proteins) are enveloped by the agglutinin and are thus transformed into small clumps of a hydrophobe colloids. The second stage is the flocculation of the hydrophobe colloid by the electrolyte in the solution. In the complete absence of salts there is an adsorption of the agglutinin but the bacteria are not thrown down as a precipitate.⁷

5. PROPERTIES OF HYDROPHILE COLLOIDS

It has already been mentioned that the hydrophile colloids, to which proteins, lecithin and other important components of protoplasm belong, occur mostly in molecular solutions and therefore lower the surface tension and raise the internal friction. For this reason they show the Tyndall effect only indistinctly and often produce an osmotic pressure in obedience to the usual laws of osmosis. The swelling of hydrophile colloids is very interesting from

¹ Freundlich. *Kolloid-Zeitschr.* 1: 321. 1907; *Zeitschr. f. physikal. Chem.* 73: 385. 1910; Freundlich und Schucht. *Ibid.* 85: 641. 1913; Jshizaka. *Ibid.* 83: 97. 1913; Gann. *Kolloidchem. Beihefte.* 8: 64. 1916.

² Smoluchowski. *Kolloid-Zeitschr.* 21: 98. 1917; Zsigmondy. *Zeitschr. f. physikal. Chem.* 92: 600. 1918; Westgren und Reitstötter. *Ibid.* 92: 750. 1918.

³ Powis. *Zeitschr. f. physikal. Chem.* 89: 91. 1915; Kruyt. *Kolloid-Zeitschr.* 22: 81. 1918; Kruyt und van der Speck. *Ibid.* 25: 1. 1919.

⁴ Linder and Picton. *Jour. Chem. Soc. (London)* 71: 572. 1897; Henri and others. *Compt. rend. soc. de biol.* 55: 1666. 1903; and others.

⁵ Billiter. *Zeitschr. f. physikal. Chem.* 51: 129. 1905; Buxton, Shaffer und Teague. *Ibid.* 57: 47 and 64. 1916.

⁶ Michaelis und Pincussohn. *Biochem. Zeitschr.* 2: 251. 1906.

⁷ Bordet. *Ann. Inst. Pasteur.* 13: 225. 1899.

the physiological standpoint. This process is often called "absorption" and must be sharply distinguished from "adsorption" since the one has nothing to do with the other.

At high concentrations, various dissolved substances increase the osmotic pressure out of proportion to the expectancy according to the theory of solutions. The hydrophile colloids develop a very high osmotic pressure whenever the ratio between the amounts of colloid and water is such that the mixture forms a solid or gelatinous body. In these cases we do not speak of a solution of the colloid in water but of a solution of water in the colloid. This is the phenomenon of swelling. It would hardly be correct to make a distinction between osmotic pressure and swelling pressure in principle. Rather we may suppose that the dissolved particles of the hydrophile colloid are swollen and so retain large amounts of water. Hence the whole system is like an emulsion (a mixture of two immiscible liquids). It is evident that we are here dealing not with a boundary surface phenomenon as in adsorption but with an even distribution of the water between the colloidal particles.¹

If a hydrophile colloid in the dry condition be moistened with water, the first water to be taken up gives rise to an enormous pressure.² The osmotic pressure of solutions reaches 100 atmospheres only in exceptional cases but the swelling of starch has given measured pressures of over 2000 atmospheres. Just as the addition of a small amount of water to anhydrous sulphuric acid or to absolute alcohol brings about the development of heat and a reduction in volume, so the swelling of a dry hydrophile colloid is attended by contraction and a considerable gain in heat.³ These phenomena may be explained satisfactorily on the basis of the hydrate theory of solutions (see below).

The powerful forces of swelling seem to play an important rôle in growth processes and in the ascent of sap in plants. There are records of the splitting and raising of enormous rocks through the action of swelling plant organs. On the basis of recent studies, the effect of acids, alkalis and salts upon the osmotic pressure and the swelling of hydrophile colloids is likewise proving very interesting. These phenomena will be discussed in another volume.

Like hydrophobe colloids, the hydrophile colloids are flocculated by electrolytes but if they have not been first changed into hydrophobe colloids (which is seldom necessary) the circumstances of the precipitation are very peculiar. The hydrophobe colloids are coagulated as the result of the removal of the electric charge; this may be done by small amounts of electrolytes. On the contrary, the hydrophile colloids are precipitated only by large quantities of salt and the process is called "salting out." This salting out process is a very widespread phenomenon outside the field of colloidal chemistry. It is known that, under the influence of neutral salts, the water solubility of alcohol, ether and other organic substances and of various gases is reduced.

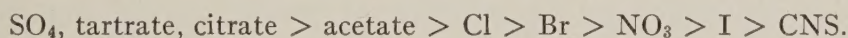
¹ Katz, J. R. *Zeitschr. f. Electrochem.* 17: 800. 1911; *Kolloid-chem. Beihefte.* 9: 1. 1918.

² Reinke, J. *Hanst. bot. Abh.* 4: 1. 1879; Rodewald. *Zeitschr. f. physikal. Chem.* 24: 206, 1897; Rodewald und Kattein. *Ibid.* 33: 586. 1900.

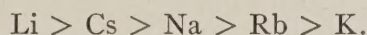
³ Katz. *loc. cit.*; cf. also Wiedemann und Lüdeking. *Ann. d. Physik.* 25: 145. 1885.

The changes in solubility of surface active substances, which include hydrophile colloids, can scarcely be measured by the use of the stalagmometer (page 2). In general the reduction of the solubility of a hydrophile colloid is accompanied by an alteration of all the properties which are functions of increased or reduced solubility. The surface tension increases but the internal resistance becomes less. The formation of gels (which occurs often in colloids) is promoted.¹ Swelling and osmotic pressure (which are really the same phenomenon as explained above) are lowered.² The effect of electrolytes may thus be recognized after the alteration of one or more of these properties which are to be regarded as functions of the solubility. The results are always the same.

The most important conclusion to be drawn from these facts is that every effect of an electrolyte may be traced to the effect of ions. In experiments with proteins, the cations of salts are less significant than the anions. If the latter are arranged in a definite series according to the salting out effect,³ the members at the extreme right of the series do not reduce the solubility of protein colloids; rather they seem to act in the opposite direction.



The cations form a less marked series; for example, the alkali metals salt out in the following order:



The salting out of proteins and other hydrophile colloids is a reversible process. If the electrolyte is removed by dialysis or through the lowering of its concentration by dilution, the colloid redissolves without changing its properties.

It must be emphasized that the unequal effect of different ions does not manifest itself alone in the salting out of colloids; these so-called "lyotropic" series appear in the salting out of various other substances from their aqueous solutions.⁴

The reduction in the solubility of various substances under the influence of electrolytes cannot be explained on the basis of the van't Hoff theory of solutions. On the whole, these facts also contradict the Henry-Dalton law. The most fruitful theory in this connection is that of Mendelejew. It is based on the affinity of various substances for water and on the formation of complex compounds containing water. At one time such views would have been regarded as antiquated. At present the hydrate theory of Jones and other chemists has just been worked out.⁵ This theory does not preclude the simultaneous existence of the van't Hoff theory. For example, the anomalous lowering of the freezing point of concentrated solutions of many salts, which stands in direct contradiction to the van't Hoff theory, is easily explained by the Jones theory. The amount of solvent is reduced by the hydrate formation and the solution is really more concentrated than one should expect from a theoretical calculation. Proteins and other hydrophile colloids are salted out likewise because the salts hold a large number of water molecules and the colloid then finds itself in a supersaturated solution.

It is impossible to consider further details of the modern theories of hydration at this point. It can only be mentioned that the unequal power of attraction of different ions can be explained from the standpoint of the hydration theory as follows. On the basis of the current

¹ **Pascheles.** *Pflügers Arch. f. ges. Physiol.* **71**: 333. 1898; **Pauli und Rona.** *Hofmeisters Beitr.* **2**: 1. 1902; **v. Schroeder.** *Zeitschr. f. physikal. Chem.* **45**: 75. 1903; **Traube und Köhler.** *Internat. Zeitschr. f. physikal.-chem. Biol.* **2**: 42. 1915; **Höber.** *Pflügers Arch. f. ges. Physiol.* **166**: 602. 1917.

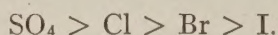
² **Lillie, R. S.** *Amer. Jour. of Physiol.* **20**: 127. 1907; **Moore and Roaf.** *Biochem. Jour.* **2**: 34. 1907; **Roaf.** *Quart. Jour. of Exp. Physiol.* **3**: 75. 1910.

³ **Hofmeister, F.** *Arch. f. exp. Pathol.* **25**: 13. 1888; **28**: 210. 1891; **Spiro.** *Hofmeisters Beitr.* **5**: 276. 1904. Loeb claims that these series of ions are based on experimental errors and do not really exist but a conclusion of this kind contradicts many well established facts; *cf.* **Loeb, J.** *Jour. Gen. Physiol.* **1**: 559. 1919; **3**: 85. 1920; **3**: 391. 1921.

⁴ **Setschenow.** *Zeitschr. f. physikal. Chem.* **4**: 117. 1889; **v. Euler.** *Ibid.* **31**: 360. 1899; **49**: 303. 1904; and many others.

⁵ **Jones.** *Zeitschr. f. physikal. Chem.* **49**: 385. 1904; **52**: 231. 1905; **Washburn.** *Jour. Amer. Chem. Soc.* **31**: 322. 1909; **Remy.** *Zeitschr. f. physikal. Chem.* **89**: 529. 1914.

notions of atomic structure, the affinity of the ions for water must be directly proportional to the atomic radius.¹ The following series of the power to attract water is thus obtained:



This corresponds to the lyotropic series.

However, there is still another explanation of the existence of the lyotropic series but it applies only to the salting out of proteins. It is based on the capacity of amino acids to form complex compounds with mineral salts. The degree of affinity of the salts to the amino acids corresponds with the order of the lyotropic series.²

All the above explanations of the effect of salts on the solubility of hydrophile colloids dealt with neutral solutions. In the presence of small amounts of acid or alkali some colloids, including proteins, have the properties of hydrophobe colloids in that they carry a definite charge. Hence the neutral salts precipitate proteins much more easily in weakly acid or alkaline solution than at a neutral reaction,³ and the precipitate does not redissolve after the salt is removed.

The lyotropic series continues to hold but it is worthy of note that in acid reaction the order of the ions in the series is the reverse of that in alkaline reaction. For example, in the precipitation of the protein of pine seedlings the following results were obtained:⁴

In acid reaction: $\text{NaCl} < \text{NaBr} < \text{NaNO}_3 < \text{NaI}$

In alkaline reaction: $\text{NaCl} > \text{NaBr} > \text{NaNO}_3, \text{NaI}.$

In alkaline reaction the last two salts precipitate practically no protein.

In many cases merely changing the concentration of the salt produces an inversion of the lyotropic series.

On the other hand, the action of salts of the heavy metals on hydrophile colloids calls to mind the effect of all salts on hydrophobe colloids. The cation is always the active factor and the precipitate cannot be returned to solution without changing the most important properties of the given colloid.⁵

Here also, as in the precipitation of the hydrophobe colloids, the electrical charge of the cations plays the important rôle. Non-electrolytes scarcely affect the hydrophobe colloids but many of them (alcohol, acetone, etc.) precipitate the hydrophile colloids. Surface-active organic substances in general cause the solutions to become gels because they reduce the solubility of the colloid. Substances which are not surface-active act in the opposite manner.

The change of colloidal solutions to gels has been mentioned repeatedly. Apparently this very peculiar process has a great physiological significance but the colloidal chemistry of it has not yet been satisfactorily cleared up. The colloids which strongly increase the internal friction of solutions tend to promote the formation of gels. Indeed it is advisable to consider the formation of gels from the standpoint of the hydration theory of solutions. The colloidal molecules take up such large quantities of water that most of the solvent is thereby claimed and the result is not only a solution of the colloid in water but

¹ Fajans. *Naturwissenschaften*. 9: 729. 1921.

² Pfeiffer, P. und v. Modelski. *Zeitschr. f. physiol. Chem.* 81: 329. 1912; 85: 1. 1913; Pfeiffer, P. und Würzler. *Ibid.* 97: 128. 1916.

³ Pauli. *Hofmeisters Beitr.* 5: 27. 1903.

⁴ Posternak. *Ann. Inst. Pasteur*. 15: 85. 1901; Pauli. *loc. cit.*; Höber. *Hofmeisters Beitr.* 11: 35. 1907; Pauli und Falek. *Biochem. Zeitschr.* 47: 269. 1912; and many others.

⁵ Pauli. *Hofmeisters Beitr.* 6: 233. 1905; 7: 531. 1906; Porges und Neubauer. *Biochem. Zeitschr.* 7: 152. 1907; Galeotti. *Zeitschr. f. physiol. Chem.* 40: 492. 1904.

also and simultaneously a solution of water in the colloid. A combination of these two phases gives a gel. Through the action of a salt, the formation of gel is either increased or retarded according to the specific effect of the various ions.¹ Previously it has been thought that a certain meshlike or honeycomb structure was peculiar to the gels but more recently it has been shown that the gel is often just as homogeneous as the solution from which it came.² Homogeneous gels are produced from concentrated solutions without a preliminary disintegration since in these cases all the water is used in the formation of hydrates. However, there are other gels which resemble a crystalline mass, the pores of which are filled with the solvent.³ Between these two extreme cases of gel formation there are various transitional types.

Different colloids exert interesting effects upon each other if one is hydrophile and the other hydrophobe. It has already been mentioned that a hydrophile colloid is often adsorbed to the surface of the hydrophobe type and the latter is then protected from flocculation. The tiny particles of the hydrophobe colloid are inclosed by a layer of hydrophile colloid and in this way protected from precipitation by electrolytes. The hydrophobe colloid thus comes to have the properties of the hydrophile colloids. In such cases the hydrophile colloid is called a "protective colloid."⁴ Protective colloids can even keep in solution a true powdery suspension. For example, relatively insoluble calcium salts go into solution if they are inclosed by a layer of hydrophile colloid.⁵ It is thought that calcium phosphate is thus included in milk.

The protective effect of colloids is quantitatively measured by the milligrams of colloid which are just necessary to prevent the change of red gold sol to violet after the addition of a certain amount of a 10% NaCl solution (the gold number).

Although the fundamental traits of capillary chemistry and the properties of colloidal solutions have been described as concisely as possible, their significance for the correct understanding of the various chemical processes in living tissues must be clear to the layman. Living cells have a heterogeneous system with an enormous development of surfaces. Colloidal substances are the principal components of protoplasm. It is not strange that older writers, who knew nothing of the recent developments of physical chemistry, were forced to report new and apparently paradoxical facts at every step, since they could attempt to explain biochemical processes on the basis of no laws other than those which held for aqueous solutions of crystalline substances. Now the modern scientific literature of physiology cannot be understood without a knowledge of the results of colloidal chemistry.

¹ Kostytschew, S. *Zeitschr. f. physiol. Chem.* 39: 545. 1903; Samec. *Kolloidchem. Beihefte.* 3: 1. 1912.

² Menz. *Zeitschr. f. physikal. Chem.* 66: 129. 1908; v. Weimarn, P. *Kolloid-Zeitschr.* 4: 133. 1909; 6: 277. 1910; Bachmann. *Ibid.* 9: 312. 1911; Idem. *Zeitschr. f. anorg. Chem.* 73: 125. 1912; Zsigmondy. *Kolloid-Zeitschr.* 11: 145. 1912.

³ Bachmann. *loc. cit.*; Flade. *Zeitschr. f. anorg. Chem.* 82: 173. 1913; Stübel. *Pflügers Arch. f. ges. Physiol.* 156: 374. 1914.

⁴ Bechhold. *Zeitschr. f. physikal. Chem.* 48: 385. 1904.

⁵ Pauli und Samec. *Biochem. Zeitschr.* 17: 235. 1909.

6. MOLECULAR ASYMMETRY AND ITS PHYSIOLOGICAL SIGNIFICANCE

To explain all the phenomena of isomers among organic compounds, it has been found necessary to develop the conception of the spatial arrangement of atoms in the molecule although equally satisfactory results would be obtained if one thought of the carbon atom in the center of a tetrahedron with the four valences of the carbon at the four corners, at which are placed four atoms or groups of atoms.¹ From Fig. 3 it is evident that if only two of the four groups at the corners of the tetrahedron are alike, this makes possible a plane of symmetry which divides the molecule into two equal halves. This plane of symmetry passes through the edge *ac* and the middle of the faces *abb* and *bbc*. If all four groups are different there can be no plane of symmetry in the molecule.

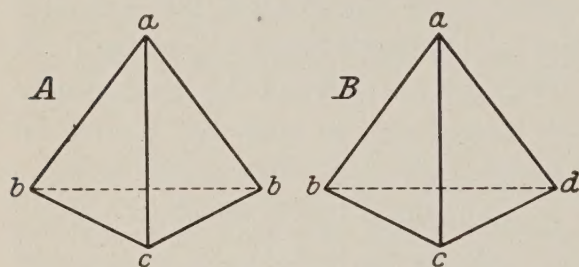


FIG. 3.—A. Symmetrical carbon tetrahedrons. The symmetry is limited to the identity of the two surfaces *abc* right and left. B. Asymmetrical carbon tetrahedrons. The four groups bound to the carbon atom are all different; hence no symmetry is possible.

Hence, a carbon atom which is surrounded by four different groups is said to be “asymmetrical” and in the structural formula is indicated by an asterisk.

If the symmetrical tetrahedron *abbc* is viewed in a mirror the image is found to be identical with the original; but if the asymmetrical one *abcd* is placed before a mirror the image is not identical because the parts on the right appear on the left and vice versa.

Such “mirror images” are often found in nature. The right hand is a mirror image of the left and a screw with a spiral to the right is a mirror image of one with a spiral to the left, etc. The pioneer work of Pasteur² showed that mirror images are also found among organic compounds and that all asymmetrical molecules exist in two different modifications. The properties of these are often identical except for the direction in which they rotate the plane of polarized light. All compounds with an asymmetrical carbon atom are optically active *i.e.*, they rotate the plane of polarized light by a certain number of degrees, even when in solution. This is an important constant of the asymmetrical molecule.³

One optically isomeric substance rotates the plane of polarized light to the right, the other to the left and by exactly the same number of degrees. The first

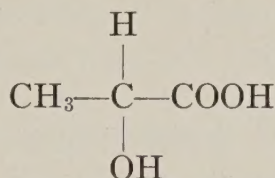
¹ van't Hoff, J. H. *La chimie dans l'espace*. 1875; Le Bel. *Bull. Soc. Chim. de France* (2) 22: 337. 1874; (3) 3: 788. 1890; 7: 613. 1892; *Idem*. *Compt. rend.* 114: 304. 1892. According to the most recent views, the single atoms and groups of atoms are not fixed in the molecule so that the corners of the tetrahedron represent only the centers of equilibrium about which the periodic movements of atoms or groups of atoms take place.

² Pasteur, L. *Ann. de chim. et de physique* (3) 28: 56. 1850; *Idem*. *Liebigs Ann. de Chem.* 88: 213. 1853; *Idem*. *Compt. rend.* 46: 616. 1858; 51: 298. 1860; and many others.

³ There are also optically active compounds which contain no asymmetrical carbon atoms. The infrequent cases have no significance for biochemistry and are not described here. The details can be found in the complete discussions by Werner, A. *Lehrbuch der Stereochemie*. 1904; van't Hoff. *Dix années dans l'histoire d'une théorie*. 1887; Meyer, V. *Ber. chem. Ges.* 23: 567. 1890.

is designated by *d* and the second by *l*.¹ The combination of the two substances in equi-molecular proportions is "optically inactive" and does not rotate polarized light. It is given the symbol "*dl*" and can be either a simple mixture or a complex chemical compound (such as racemic acid, the optically inactive form of tartaric acid). In this case the substance is said to be "racemic."

Up to this point we have considered only one asymmetrical carbon atom in the molecule. Lactic acid is a substance of this type:



If there are more asymmetrical carbon atoms in the molecule we may have spatial isomerism. Let us consider a pair of asymmetrical carbon atoms united together. Let the one be united to the three groups *a*, *b* and *c* and the second to the groups *d*, *e* and *f* in addition to the common union. Theoretically, the following spatial arrangements of the atoms in the molecule are possible (Fig. 4).

It is evident that nos. 1 and 2 are optical mirror images or, as they are called for brevity, *enantiomers*. They differ only in the direction of the rotation of polarized light. The chemical properties are identical. The same thing is true of nos. 3 and 4; they are also enantiomers. On the contrary, there is a noticeable difference between the two pairs (1, 2) and (3, 4). The interval between single groups of the upper and lower tetrahedrons (*e.g.*, between *c* and *f*) is not the same in the two pairs of figures. Hence, the chemical properties of these substances must

be different and the difference can be experimentally established. These isomers are called *diaisomers*; they differ from each other by various chemical properties and by the size of the angle of rotation of the plane of polarized light.

The stereoisomers of cyclic compounds are constructed on the same principle (*cis*- and *trans*- forms) and we shall not describe them here.

In order to simplify matters, the stereoisomers are usually represented as projections. This can be easily understood by comparing the figures in Fig. 4

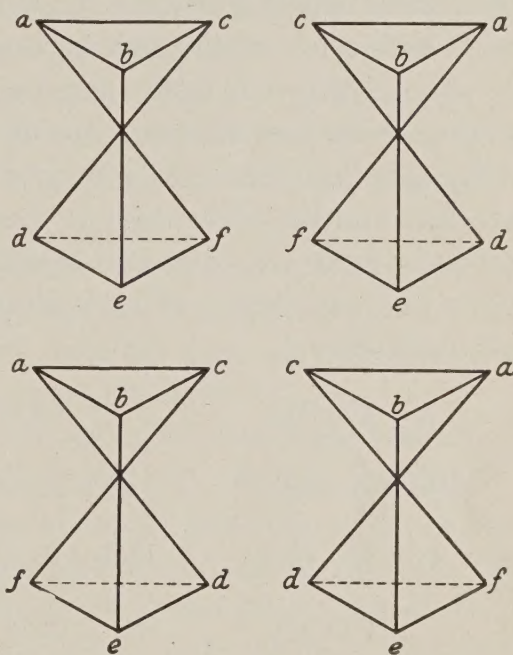
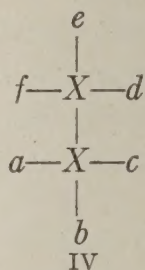
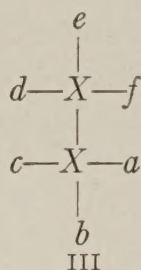
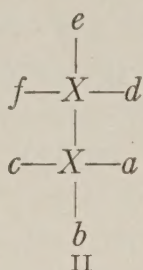
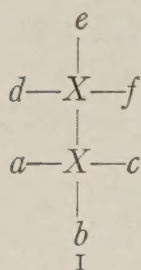


FIG. 4.—Schematic structure of diaisomers and enantiomers (explanation in the text).

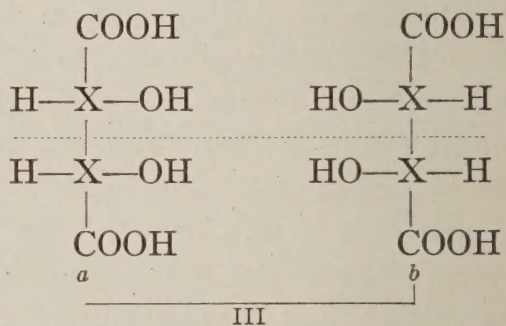
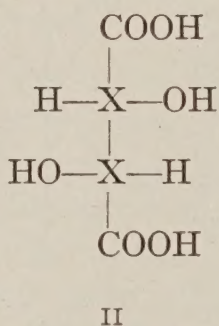
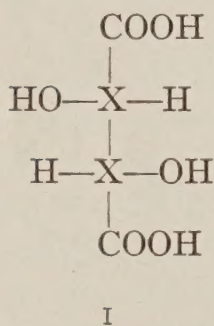
¹ The direction of rotation does not always correspond to these symbols. For example, if a substance A, which rotates the light to the right, and a substance B which rotates the light to the left are genetically related, the latter is called *d* and the other one *l*. Aside from the direction of the rotation, optical isomers differ from each other in the solid condition through their crystalline form. The asymmetrical crystals of *d*- and *l*-substances are always mirror images yet the position of the hemihedral faces does not always correspond to the rotation symbol, as Pasteur showed.

with the following projection formulae. In the latter the carbon atom is indicated by a cross X:



If there are n asymmetrical carbon atoms, united with different atomic groups, contained in a molecule, the number of possible stereoisomers is 2^n ; but if some of the atoms or groups are duplicated the possible number of isomers is thereby reduced. It is evident that it is unimportant for the total number of isomers whether all the asymmetrical carbon atoms are connected directly with each other or through an intermediate member.

Hence, if two or more asymmetrical carbon atoms are included in a molecule, there are diastereoisomers in addition to the enantioisomers. This is the first difference between such molecules and those with only one asymmetrical carbon atom. Another difference is found in the cases in which the asymmetrical carbon atoms are united to identical groups of atoms. Thus for tartaric acid with two asymmetrical carbon atoms there are the following possible spatial arrangements:



Numbers I and II are "mirror images" or enantioisomers but numbers IIIa and IIIb represent the same optically inactive substance because the optical rotation of the two tetrahedrons takes place within the molecule in opposite directions. If all the formulae are borne in mind, it will be found that numbers I and II show no changes but that IIIa changes to IIIb and vice versa. Hence IIIa and IIIb are only two different representations of a single projection formula. The object and its mirror image are identical. As explained previously, this can only mean the existence of a plane of symmetry. Although each carbon atom of itself is asymmetrical, still the molecule has the plane of symmetry shown by a dotted line through the formula and the substance is therefore optically inactive.

Stereoisomerism plays a prominent rôle in plant nutrition. As Pasteur discovered, all physiologically important substances are asymmetrical. Proto-

plasm itself consists of optically active asymmetrical substances and produces only such. According to Pasteur, life as a whole is a result of the action of asymmetrical forces. For organisms, the differences between diastomers and even between enantiomers are often more significant than those between substances of different chemical composition, *e.g.*, the adjacent members of a homologous series. Yeast readily changes large amounts of *d*-glucose to alcohol and carbon dioxide but is unable to ferment the various diastomers of glucose or even its mirror image—*l*-glucose. On the contrary, *d*-fructose is attacked by yeast although this sugar has a structure quite unlike that of glucose and its stereoisomers. No living cell is able to synthesize proteins from optical isomers of naturally occurring amino acids. Many molds and bacteria do well on *d*-tartaric acid and *l*-malic acid but do not grow properly when fed with the optical antipodes of these acids and even prefer substances of entirely different composition. From these facts it must be concluded that optical isomers differ from one another not only in the rotation of polarized light but also in certain unknown properties which are biologically important.

As Pasteur correctly put it, the riddle of life cannot be solved until we have a thorough knowledge of the nature of the asymmetrical forces and spatial isomerism. It is also quite possible that without this knowledge even enzyme action cannot be understood (see below), since enzymes are very sensitive to optical isomers. And all the important physiological transformations of materials are dependent upon enzyme action. That optically active substances are found exclusively in nature is more important than the fact that they can be produced in the laboratory under certain restricted conditions.

However, this is entirely conceivable because the synthesis of asymmetrical compounds is nothing but a change of $A(abbc)$ to $B(abcd)$ (Fig. 3). From the figure it is clear that the two *b* groups occupy identical positions with respect to the other parts of the molecule and are placed at equal distances from the carbon atom and the other groups. Hence there is no reason that one of the *b* groups should be replaced by the group *d* in a greater number of molecules than the other groups. It is rather to be assumed according to the theory of probability that the right and left isomeric substances are formed in equal amounts and that the mixture of them forms an inactive *dl*-substance. Actually, these theoretical assumptions are supported by a large amount of experimental evidence. Outside of living cells only the *dl*-inactive asymmetrical substances are synthesized from symmetrical substances.

Still it is possible to isolate from such mixtures one, or in many cases, both the optically active substances by suitable methods. Three methods for this purpose were worked out by Pasteur¹ 70 years ago and no newer devices have been found since. The first method consists in the mechanical separation of the crystals. As already mentioned, the left and right rotation is often correlated with the left and right hemihedrism of crystals. Through careful selection of the crystals by means of a magnifying lens, it is possible to separate the enantiomers. This method, which led to the discovery of molecular asymmetry, applies only to substances which form large, well-defined crystals. Pasteur soon replaced it with other methods.

¹ Pasteur, L. *loc. cit.*

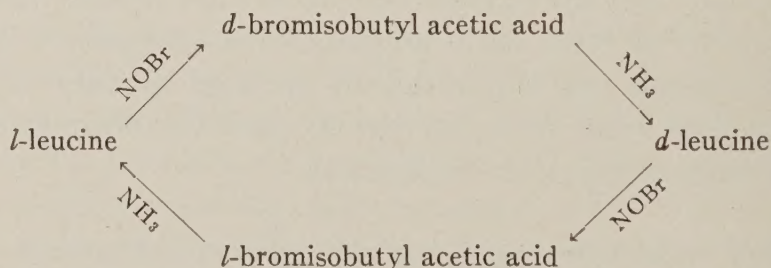
The second method consists in the utilization of the mysterious asymmetrical forces of organic nature itself. Some microorganisms take from a solution of *dl*-isomers only one optically active substance and usually the one which is found in natural products. The other optically active substance remains in the solution. By using definite and well known organisms it is possible to obtain very satisfactory results. Of course, this method cannot be applied to toxic substances (such as alkaloids) because the microorganisms cannot feed on these substances.

The third method has a general significance. The mixture of the two enantioisomeric substances is allowed to react with an optically active compound. This method is especially valuable for acids and bases which form optically active salts. For the optically active acid it is customary to use the *d*-tartaric acid taken from plants. For the optically active base, one chooses an alkaloid (strychnine, brucine or quinine) isolated from plants. The method is based on the fact that the following combinations are possible in the union of optically active bases with optically active acids.

1. *d*-base and *d*-acid.
2. *l*-base and *l*-acid.
3. *d*-base and *l*-acid.
4. *l*-base and *d*-acid.

The same considerations could be applied to these salts as to the compounds with two asymmetrical carbon atoms. Figure 4 illustrates it. The salts 1 and 2 are enantioisomers and 3 and 4 are likewise. On the contrary the pairs (1 & 2) and (3 & 4) are diastereoisomers. These pairs must differ from each other by angle of rotation, solubility and other properties. Let us suppose now that we are joining *d*-tartaric acid with an inactive *dl*-base. Out of the four possibilities only two could occur in this case—1 and 4 which include the *d*-acid. But these salts are diastomers and can be separated on the basis of their different solubilities and other properties. Then the separate salts can be decomposed and the optically active *d*- and *l*-bases isolated. In practice it is often necessary to prepare only one optically active substance because one enantioisomer can be transformed into another through certain reactions which are included in the "Walden inversion." These astonishing reactions are of great interest to biologists as well as to chemists.

If the amino group in an active amino acid, such as *l*-leucine, is displaced by Br (by the action of NOBr), the *d*-bromic acid is obtained. If the bromine is again displaced by the amino group (action of NH₃), *d*-leucine is obtained. Hence the direction of rotation has been changed. If we reverse the procedure *i.e.*, start with *d*-leucine, displace its amino group with Br, and then replace the amino group, the result is *l*-leucine—the initial material. The whole cycle is shown by the scheme:

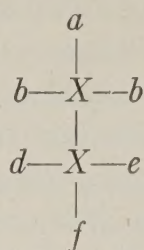


The Walden inversion in the hands of E. Fischer and his colleagues has been found to be a useful method for the preparation of various optically active amino acids.¹

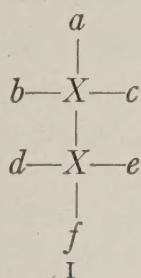
If we are unable to prepare optically active substances from optically inactive material, we can still make them from other optically active compounds. If

¹ Fischer, E. Ber. Chem. Ges. 40: 489. 1907.

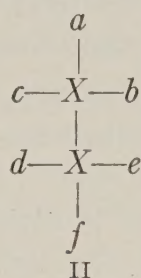
group *b* is replaced by group *c* in a compound with only one asymmetrical carbon atom, such as



the result is either

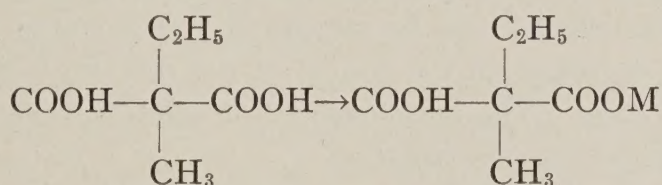


or



However, in an asymmetrical molecule the two *b* groups are not equally distant from *d* and *e* (Fig. 4). Hence one of the two possible diaisomeric products must occur in large amounts or even exclusively, because the chemical properties of diaisomers are not identical.

This case shows the possibility of an asymmetrical synthesis.¹ An optically active base *M* may form a salt with a symmetrically formed acid and the symmetry of the latter be lost. For example:



On account of the asymmetry of *M* we have here a case entirely analogous to the one mentioned above. The two isomers must be found in unequal amounts. After *M* and CO_2 are removed, we obtained an optically active monobasic acid from the optically inactive dibasic acid. On the contrary, if we split CO_2 directly from the substance $\text{COOH}-\text{C}(\text{CH}_3)-\text{C}_2\text{H}_5-\text{COOH}$ the result is a *dl*-inactive product. According to the view of many investigators,² such processes may explain the formation of optically active substances in the organism. That is, it is assumed that the nutrient substances enter into compounds by being worked up with various asymmetrical complexes *e.g.*, with proteins, phosphatides and especially enzymes. The well known specificity of enzymes for the optically active substances is made clear by their own asymmetrical structure.

Of course, it cannot be denied that these processes are quite probable but it is to be noted that such an hypothesis is clumsy because it compels one to

¹ Marckwald. Ber. chem. Ges. 37: 49 and 1368. 1904; McKenzie. Jour. Chem. Soc. (London) 85: 1249. 1904.

² *E.g.*, Fischer, E. Ber. chem. Ges. 27: 3230. 1894.

accumulate unproven assumptions. The supposed need for union between every nutrient and some enzyme depends *a priori* upon the theory of catalysis and indeed upon the theory of intermediate products. A new problem also arises: if each asymmetrical carbon atom is produced from a previous one, it is not quite conceivable how the first asymmetrical carbon atom was formed on the earth. The riddle is analogous to that of the origin of life on earth. One seeks to solve it by drawing in circum-polarized light.¹

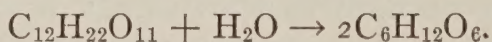
However, such complicated hypotheses are perhaps unnecessary because molecular asymmetry is so easily formed in organisms. Some investigators are inclined to the belief that special asymmetrical forces are at work in protoplasm and that they bring about asymmetrical syntheses. According to this idea, various substances are transformed into the corresponding stereoisomers previous to the synthesis.² This theory is supported by proofs which we do not need to consider here.³

In conclusion, it should be noticed that some writers deny the significance of asymmetrical cleavages and syntheses for vital phenomena.⁴ The specificity of the organism for definite stereoisomers is explained as a "fixed habit" of organisms. One hypothesis is thereby built on another. From the biochemical standpoint, what is the meaning of "the habit of the organism"? The question cannot be answered at the present time.

7. VELOCITY OF CHEMICAL REACTIONS

Reactions between ions take place in solutions of molecules with velocities too great to measure. On the contrary, reactions of non-electrolytes proceed so slowly that their velocity can be measured. It is found that the velocity either increases or decreases in the course of the reaction and that it depends upon various conditions. The measurement of reaction velocities is an invaluable service to biochemical investigation.

We shall first consider the simplest case in which a single substance is broken down and two other substances are formed. An example of this type of reaction is found in the hydrolysis (inversion) of cane sugar which gives rise to the simple sugars glucose and fructose.



Although two substances—sugar and water—apparently take part in this reaction, the quantity of water involved is such that the amount of it used up need not be considered. For reasons to be discussed later, in this case one is entitled to speak of it as the cleavage of one substance—sugar—in so far as the reaction velocity is concerned. According to the law of mass action, the velocity

¹ Byk. Zeitschr. f. physikal. Chem. 49: 641. 1904; Pirak. Biochem. Zeitschr. 130: 76. 1922; and others.

² Erlenmeyer, E. Biochem. Zeitschr. 35: 144. 1911; 67: 346. 1914; 97: 198, 245, 255 and 261. 1919.

³ Other facts favor this view. For example, the simple reduction of $\text{CH}_3\text{—CO—CH}_2\text{OH}$ by yeast produces the optically active propylene glycol, $\text{CH}_3\text{—CHOH—CH}_2\text{OH}$; cf. Färber, E., F. F. Nord und C. Neuberg. Biochem. Zeitschr. 112: 313. 1920. Here there is only an addition of hydrogen and not a synthesis but the result is an asymmetrical product.

⁴ Condelli, S. Gazz. chim. ital. 51(II): 309. 1921.

is proportional to the concentration of the active substance. At first sight it would seem that the measurement of the velocity could be made by noting the concentration of sugar at the start and at the end of an experiment, the time of which is known exactly, and by calculating the amount per unit of time. However, such a rough measurement does not yield comparable data for different reactions because the concentration of decomposing substance is constantly decreasing. As time passes arithmetically, the concentration decreases logarithmically *i.e.*, rapidly at first, then more and more slowly and becomes zero in infinite time. Such a process is graphically represented by means of an asymptotic curve (Fig. 5), for which there is a simple differential equation.

If we take the amount of substance changed as dx in the time dt , the reaction velocity is expressed as $\frac{dx}{dt}$. Let the molar concentration of the substance be equal to c at the start of the reaction. Then after the expiration of t units of time the concentration will be $c - x$ if x molecules of the substances are decomposed. The differential equation of the reaction velocity will then be

$$\frac{dx}{dt} = k(c - x).$$

This equation is easily understood because it represents the dependence of the reaction velocity upon the concentration of the substance $c - x$ at a particular moment. k is a constant which is characteristic of the substance concerned in the reaction. It expresses the reaction velocity

of the theoretically unchanged concentration of one mol per liter. From the equation it is evident that the velocity constantly decreases and becomes too small to measure as $c - x$ approaches 0. This is shown by the curve in Fig. 5. It is also evident that when the quantity of the substance x is almost equal (in mols) to the entire amount of the substance c with which the experiment was begun, $c - x$ and consequently the whole right side of the equation is infinitely small.

It is evident that the determination of the constant k gives an accurate and clear conception of the velocity of any reaction. It not only provides for the comparison of reactions but also makes it possible to investigate changes in velocity of a given reaction under the influence of various catalysts (*i.e.*, factors which govern the velocities of chemical processes). This is very significant for chemical physiology since the most important material transformations in plants are catalyzed processes.

The first determinations of the velocity constants were carried out in 1850 upon the inversion of cane sugar in the presence of acids.¹

Of course the integral equation must be used to determine the velocity constant of chemical reactions. If the foregoing equation

$$\frac{dx}{dt} = k(c - x)$$

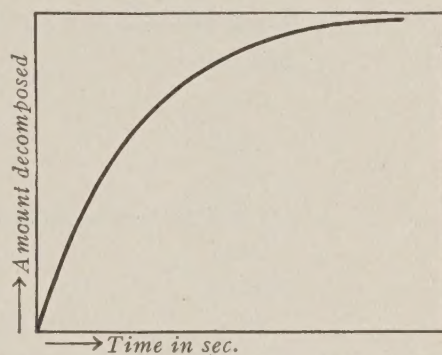


FIG. 5.—Asymptotic curve of the velocity of a chemical reaction.

¹ Wilhelmy, L. Pogg. Ann. 81: 413. 1850.

be integrated the result is

$$kt = \ln \frac{c}{c-x},$$

in which $\ln$ indicates natural logarithms. From this equation it can be seen directly that as time increases in arithmetical progression, the concentration decreases logarithmically. If, for convenience, the natural logarithms are to be changed to common logarithms, there is for k an expression in which all terms can be easily determined by experiments:

$$k = \frac{1}{0.4343t} \log_{10} \frac{c}{c-x}$$

Hence it is only necessary to make accurate measurements of the intervals of time at which portions of the solution are taken for quantitative determination (by the most rapid methods—by polarimeter, colorimeter, titration, etc.) of the molecular concentration $c-x$. In addition, the molar concentration c at the start of the experiment is exactly measured. The values are then substituted in the equation. The following table, taken from Nernst's book (*Theoretische Chemie*, 7th Ed. 1913), serves as an example. It represents a determination of the inversion constant of a 20% cane sugar solution in the presence of 0.5 N lactic acid at 25° C.

Time in Seconds	Rotation of Polarized Light	Constant k
	+34.5° (c)	
1435	+31.1°	0.235
4315	+25.0°	0.236
11360	+13.98°	0.231
16935	+ 7.57°	0.232
29925	- 1.65°	0.233
∞	-10.77°	

This table shows the accuracy with which the constant can be determined.

The reaction just described, in which a single substance is decomposed, is called a monomolecular reaction or a reaction of the first order. In organisms we also find bimolecular reactions or reactions of the second order, in which two substances are involved simultaneously. Reactions of the third order are very seldom found in chemistry and they never concern biochemistry. Reactions of a still higher order are practically non-existent. There are some chemical equations which, at first sight, express such processes but they are always found to be combinations of reactions.

The saponification of ethyl acetate by alkali is an example of a reaction of the second order:



The differential dx requires the molecular concentrations of both ester and caustic soda and to be applicable to all cases the equation is written

$$\frac{dx}{dt} = k(c_1 - x)(c_2 - x)$$

where c_1 and c_2 are the molecular concentrations of ethyl acetate and alkali at the start of the experiment. If, for simplicity, we make $c_1 = c_2 = c$ the equation takes the form

$$\frac{dx}{dt} = k(c - x)^2$$

and after integration

$$k = \frac{x}{ct(c - x)}$$

In contrast with reactions of the first order, the constant of reactions of the second order is dependent upon both time and the original concentration c . It is evident that the same experimental data are needed to determine the constant for reactions of both the first and second order *i.e.*, determinations of the time t and the molecular concentrations c and $c - x$.

It can now be understood why the inversion of cane sugar is to be regarded as a monomolecular reaction. If it should be considered as bimolecular, $\frac{dx}{dt} = k_1(c_1 - x)(c_2 - x)$ in which c_1 is the concentration of the sugar and c_2 is the concentration of the water. But c_2 is always so much larger than x that the value of $c_2 - x$ does not change perceptibly during the experiment; hence $k_1(c_2 - x)$ is to be regarded as a constant k .

It is interesting to note the influence of temperature on the reaction velocity.¹ It is shown by the differential equation:

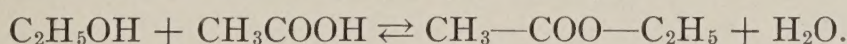
$$\frac{d \ln k}{dT} = \frac{A}{T^2} + B$$

In a somewhat simplified form² this equation becomes, after integration,

$$\ln \frac{k_1}{k_2} = \frac{A(T_1 - T_2)}{T_1 - T_2}.$$

A is a constant and T_1 and T_2 are two absolute temperatures which correspond to the velocity constants k_1 and k_2 . It appears from experimental work that for 10° intervals of temperature, the temperature coefficient $\frac{k(t + 10)}{kt}$ fluctuates between 2 and 3.5, according to the value of A . As a general rule, irregular temperature coefficients are found first at temperatures so high that they do not concern biochemical processes. To be sure, exceptions to the rule have been found and very high coefficients have been obtained in some cases.

Up to now we have considered only irreversible reactions but for many chemical processes there is an equilibrium, as soon as a certain ratio is reached, between the concentrations of the initial substances and the products that are formed. This condition goes as far as an equilibrium between two opposed reactions. For example, the formation of ethyl acetate from ethyl alcohol and acetic acid ceases when a certain concentration of the ester is reached. The same concentration also hinders the opposite process of splitting the ester. Such reversible reactions are indicated by $\rightleftharpoons$ as *e.g.*



At the start the velocity of formation of the ester is proportional to the initial concentrations of the alcohol and acid

$$v_1 = k_1 C_{\text{alc}} C_{\text{acid}}$$

¹ van't Hoff. Vorlesungen. Bd. 1, P. 229.

² Arrhenius, S. Zeitschr. f. physikal. Chem. 4: 226. 1889.

where k_1 is a constant. The opposite reaction of ester cleavage starts at the same time and proceeds with the velocity

$$v_2 = k_2 C_{\text{ester}} C_{\text{water}}.$$

Hence at any moment the velocity of the whole process is

$$v_1 - v_2 = k_1 C_{\text{alc}} C_{\text{acid}} - k_2 C_{\text{ester}} C_{\text{water}}.$$

Since the velocity v_1 continues to fall and v_2 must rise according to the increase of C_{ester} , there finally comes an equilibrium where $v_1 = v_2$. Then the reaction comes to a stand-still. This equilibrium depends upon a certain ratio of the concentrations of the substances involved in the process. If $v_1 = v_2$ then

$$k_1 C_{\text{alc}} C_{\text{acid}} = k_2 C_{\text{ester}} C_{\text{water}}$$

or

$$\frac{k_1}{k_2} = \frac{C_{\text{ester}} C_{\text{water}}}{C_{\text{alc}} C_{\text{acid}}} = K.$$

For ethyl acetate and equal molar concentrations of alcohol and acid there is equilibrium when $\frac{2}{3}$ of the alcohol and acid have combined in the ester. If we substitute these values in the equation we obtain

$$K = \frac{\frac{1}{3} \cdot \frac{1}{3}}{\frac{2}{3} \cdot \frac{2}{3}} = \frac{1}{4}.$$

The general expression for the law of mass action has the formula

$$K = \frac{C_{\text{I}}^{n_{\text{I}}} \cdot C_{\text{II}}^{n_{\text{II}}} \cdot C_{\text{III}}^{n_{\text{III}}} \cdot \dots}{C_1^{n_1} \cdot C_2^{n_2} \cdot C_3^{n_3} \cdot \dots}.$$

In this expression C_{I} , C_{II} and C_{III} are molecular concentrations of the vanishing substances and C_1 , C_2 and C_3 are the concentrations of the substances being formed by the reaction. The number of molecules of each substance taking part in the reaction is n_{I} , n_{II} , n_{III} , n_1 , n_2 and n_3 while K is the constant of the chemical equilibrium. This formula also applies for all cases of dissociation and ionization.

However, there are often cases in which there is no equilibrium as calculated according to the law of mass action, and the reaction proceeds to completion. For example, this occurs in cases in which the reaction products are removed from the reaction medium because they either take part in new reactions with other substances or pass into the solid or gaseous condition and disappear from the solution. In plants there are also processes which disturb the equilibrium of various reactions. Thus in the formation of starch from sugar, the velocity of the condensation of sugar is not diminished because the product of the reaction is insoluble.

In conclusion it should be noticed that the velocity of reactions, by analogy with Ohm's law, is the result of the counteraction of the driving force (concentration, etc.) and the passive resistance (internal friction, etc.).

$$\text{Velocity of Reaction} = \frac{\text{driving force}}{\text{passive resistance}}$$

The passive resistance can be decreased just as the friction of machinery is lowered by lubricating oils. The substances which lessen the passive resistance of chemical processes are known as "catalysts." They play an important rôle in all transformations of materials in organisms and the relationships of the velocities of the chemical reactions are very important in the explanation of catalytic processes.

All the preceding expositions refer to chemical reactions in simple, molecular solutions. The subject becomes complicated if account is taken of heterogeneous systems. In a living cell the usual medium is of course heterogeneous. Unfortunately the reaction velocities in heterogeneous systems have not been very thoroughly investigated and we know of only a few of the deviations from the laws which hold for molecular solutions.

We shall first consider the following model.¹ A dilute aqueous solution of an acid is shaken with an ester which is scarcely soluble in water. In the aqueous solution the ester is decomposed but the rate of diffusion of new supplies of the ester in aqueous solution is faster than the velocity of cleavage of the ester. So in this case the reaction proceeds with a practically constant concentration of the decomposing substance and the equation for the reaction velocity

$$\frac{dx}{dt} = k(c - x)$$

becomes $\frac{dx}{dt} = k$ because there is no decrease in the concentration c .² Of course, under such conditions the complete change of the substance is really brought on faster because in a homogeneous medium the constantly declining value of $c - x$ is always lowering the reaction velocity (p. 23). Heterogeneous colloidal solutions with their enormous development of surfaces are especially suited to ensuring transformations of different substances at a constant concentration. This must be taken into consideration in connection with the part played by enzyme action. If a substance which is being changed in one phase then passes over into another phase, the rate of diffusion in the phase from which the substance is passing is significant for the maintenance of a constant concentration. We find an analogous case in the solution of a solid in a liquid. The solid is always surrounded by a layer of saturated solution. At the boundary of the phase in which the substance is being worked over the concentration of the latter is decreasing and the rate at which it falls depends on the rate of diffusion. It is therefore evident that the law of mass action is practically inactive in heterogeneous media. We find an analogous case in the solution of a solid in a liquid. The solid is always surrounded by a layer of saturated solution. The velocity of the process of solution depends entirely on the rate of diffusion in the liquid. The equation for a monomolecular reaction can be applied here as follows:

¹ Goldschmidt. *Zeitschr. f. physikal. Chem.* 31: 235. 1899; Bodenstein. *Ibid.* 29: 315. 1899 and 46: 725. 1903.

² It was mentioned above that the constant k represents the reaction velocity at a constant concentration.

$$\frac{dx}{dt} = k(S - x),$$

where S is the concentration of saturation and x is the actual concentration at a given moment. When $S = x$, $\frac{dx}{dt} = 0$. This is the condition in a saturated solution when the rate at which the solid goes into solution is actually 0.

On account of these and other conditions, even ionic reactions proceed in heterogeneous media at velocities which are often measurable. This is of great importance in the case of weakly dissociated substances because ionization in colloidal solutions decreases as the internal friction increases.¹

8. CHEMICAL INDUCTION AND CATALYSIS

Some chemical reactions are accelerated when certain other chemical reactions are going on at the same time. Often the acceleration is so large that without it the reaction appears not to take place at all. Such phenomena are especially evident in the field of oxidations. Thus, hydrogen peroxide acts slowly on tartaric acid but it can easily oxidize ferrous to ferric salts. To a surprising degree it can thereby also oxidize tartaric acid.

In the same way, indigo white is but slowly oxidized in the air. However, it is easily oxidized in the presence of the simultaneous autoxidation of triethyl phosphine or aldehydes in atmospheric oxygen. In general we have the following condition:

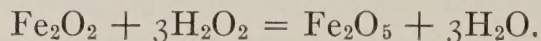
$A + O$ — reaction is possible

$B + O$ — reaction is impossible

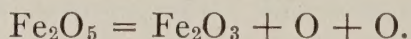
$A + B + O + O$ — both reactions $A + O$ and $B + O$ are possible.

Such reactions are said to be "coupled"² and the process is known as chemical induction.

By nature, chemical induction always causes an increase in reaction velocity because it only works on reactions which of themselves proceed very slowly. The nature of induction is the formation of intermediate products which are not always easy to detect. For example, the oxidation of tartaric acid by hydrogen peroxide is due to the fact that ferrous oxide is not oxidized directly to ferric oxide but it first becomes a peroxide:



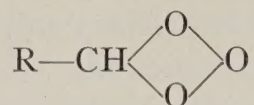
Iron peroxide has a higher oxidation potential than hydrogen peroxide and is thus able to oxidize tartaric acid. By splitting off two atoms of active oxygen, the iron peroxide becomes the ferric oxide:



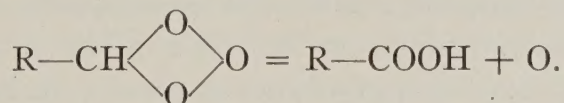
¹ Arrhenius, S. *Zeitschr. f. physikal. Chem.* 9: 487. 1892.

² Luther und Schilow. *Zeitschr. f. physikal. Chem.* 42: 641. 1903; 46: 777. 1903; Manchot. *Ber. chem. Ges.* 34: 2479. 1901; Idem. *Liebigs Ann. d. Chem.* 325: 93 and 105. 1902; Ostwald, W. *Zeitschr. f. physikal. Chem.* 34: 248. 1900; 47: 127. 1904.

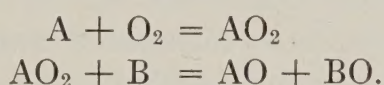
In the same way, an aldehyde $R-CHO$ absorbs two atoms of oxygen by autoxidation and becomes a peroxide:



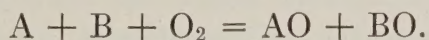
This peroxide possesses a considerably higher oxidation potential than molecular oxygen and oxidizes indigo white. The peroxide itself becomes an acid.



The general scheme of autoxidation by atmospheric oxygen may be represented as follows:

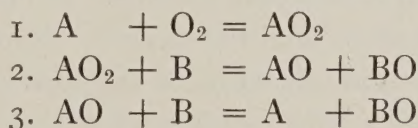


If these two equations are contracted into one the result is

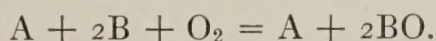


At first sight the substance B is oxidized only as the result of the simultaneous oxidation of A by the atmospheric oxygen. Investigation of the intermediate stages, however, proves that a labile peroxide AO_2 always acts as an inductor. Moreover, it is evident from these examples that coupled reactions represent a very simple form of self-regulatory processes, because the accumulation of the various products depends only on the ratio of the velocities of the separate coupled reactions. For example, if the reaction $AO_2 + A = 2AO$ goes faster than $AO_2 + B = AO + BO$, the end result will not be the same as when the second equation has the higher reaction velocity. Coupled reactions undoubtedly play an important rôle in the vital processes of organisms but they have not been thoroughly studied and only those related to biochemical oxidation have been established with certainty. So-called catalysis, a special case of chemical induction, has been studied much more.

Continuing with an example of oxidation, it is to be noted that the peroxide AO_2 which is formed in the oxidation of various substances by atmospheric oxygen, often passes on to the other bodies, not half, but all of the oxygen which it absorbs. Thus we have the following set of reactions:



If reaction 2 proceeds faster than reaction 3, an analysis at a given moment will show the presence of both AO and BO . The whole process then illustrates chemical induction. If, on the contrary, reaction 3 has a higher velocity than reaction 2 the combined result of the three equations is



In the case where the analysis shows only the original amount of unchanged A and the oxidized B, although, of itself, the latter is but very slowly oxidized, the impression is given that B is oxidized only in the presence of A although the latter takes no part in the reaction. Such substances, which seem to work only through their presence, are called "catalysts" and the process itself is called "catalysis."

Catalytic phenomena are widely distributed in organisms. At present it can be said that all the most important vital transformations take place through the agency of chemical induction or catalysis. The specific catalysts which are produced by the protoplasm of animal or plant cells are called "enzymes." It will be explained later why we can correctly consider enzymes as typical catalysts but first we shall deal with catalysis in its general aspects.

Catalytic phenomena were discovered by the great chemist Berzelius.¹ He characterized catalysts merely as substances, which by their presence alone, are able to reveal hidden chemical affinities. Today, catalysis is defined, according to Ostwald's² formulation, as follows: a catalyst changes only the rate of a chemical reaction but brings about no material transformation which cannot take place spontaneously (in the absence of the catalyst). Hence the point of equilibrium as determined by the law of mass action (p. 26) is not changed by catalytic action; the equilibrium is only reached more rapidly. Moreover, catalysts are able to act when present in very minute quantities and, in contrast with inductors, may be recovered in unchanged form when the reaction is completed.

Catalysis should not be confused with phenomena in which potential energy is liberated and changed to the kinetic form. These two categories of chemical processes have nothing to do with each other. In the release of energy there are processes which do not start of themselves but depend upon an external stimulus. As examples of such processes in daily life, there is the firing of a gun which releases the chemical energy of the components of the powder, or the movement of the locomotive throttle which puts to work the potential energy of the compressed steam. The act of liberation itself involves a loss of energy which bears no relation to the amount of work performed. It is self-evident that the force required to pull the trigger of the gun does not correspond at all to the effect of the shot while opening the throttle of the locomotive starts a very heavy train. At the same time there is a proportion, governed by the laws of thermodynamics, between the pressure of the gases in the gun barrel and the active force of the shot and between the steam pressure in the cylinder of the locomotive and the dynamic force of the moving train.

Catalysts work quite differently. They merely reduce the passive resistance of chemical reactions (p. 26) or, in other words, they are the lubricating oil in the working parts of the locomotive.³ An unoiled locomotive is unable to start a train; still it cannot be said that it is entirely incapable of doing work

¹ Berzelius, J. Lehrbuch. 3rd Ed. Vol. 6, p. 22.

² Ostwald, W. Zeitschr. f. physikal. Chem. 2: 139. 1888; 15: 706. 1894; 19: 160. 1896; 29: 190. 1899; Idem. Lehrbuch der allgem. Chem. 2nd Ed. Vol. 2, part II, p. 262. 1897.

³ Bredig, G. Ergebnisse der Physiologie for year 1902, part I, p. 136.

in this condition; but before the throttle was opened it was actually incapable of work. A very common example of catalysis is represented by the favorable effect of acids on various hydrolyses such as the saponification of an ester or the inversion of cane sugar. The first-named case is an illustration of autocatalysis *i.e.*, of a process by which the catalyst (in this case the acid or, more correctly, the hydrogen ions) continually increases and the process proceeds at an increasing rate. Such phenomena appear to be very common in organisms. However, the effect of spongy platinum on the activity of hydrogen and on other reactions is a typical catalysis. The effect of aluminium chloride on various reactions of organic substances is also analogous.

It is very interesting that all solutions of colloidal metals exert catalytic effects. Colloidal solutions of gold, platinum or silver can be easily prepared by passing an electric current between electrodes of these metals under water.¹ The result is a dark-colored, easily-filtered solution of very small internal friction and which contains a well-dispersed turbidity of tiny particles. For example, a gram atom of platinum can decompose the "solution" of hydrogen peroxide in 70 million liters of water, reduce nitrates to ammonia by means of active hydrogen, etc. The effect of these metallic catalysts is almost universal, like that of hydrogen ions. However, there are other catalysts which exert a very specific effect. Thus spongy platinum catalyses the cleavage of hydrogen from hexamethylene but it does not affect pentamethylene or hexane.² Usually the reaction velocity is increased by the action of the catalyst but the opposite effect is known *e.g.*, the effect of hydrocyanic acid on various processes, particularly on the cleavage of hydrogen peroxide.³

It is not superfluous to go somewhat further into the Ostwald theory of catalysis in order to determine whether it is actually correct in its basic principles. In the first place, it may be doubtful if a catalyst merely changes the velocity of a reaction which goes of itself. At first sight it might be thought that many reactions are started by the catalyst. However, we have at our disposal a sure method of checking the doubtful cases by means of an increase in temperature in the absence of the catalyst. As already mentioned above, determinations of the increase in reaction velocity, carried out to prove van't Hoff's equation, have shown at least a doubling of the rate for each 10°C. interval of temperature. So if a reaction velocity is equal to zero the reaction cannot be made noticeable by raising the temperature, but if a reaction is actually going on at a very low rate, the corresponding increase in temperature must make it more intensive. This is also true of reactions which are affected by catalysts. All these reactions have a noticeable velocity at sufficiently high temperatures without the aid of catalysis.⁴

¹ Bredig, G. *Anorganische Fermente*. 1901.

² Zelinski. *Ber. chem. Ges.* 44: 3121. 1911; 45: 3678. 1912.

³ Titow. *Zeitschr. f. physikal. Chem.* 45: 652. 1903; Bredig. *loc. cit.*; Kastle and Loevenhardt. *Amer. Chem. Jour.* 29: 397. 1913; and others. It might be assumed, perhaps, that the surface-active substances displace the substances adsorbed by the colloidal metal and thereby check their action. Cf. Meyerhof. *Pflügers Arch. f. ges. Physiol.* 157: 307. 1914.

⁴ For example, hydrogen reacts with oxygen with a measurable velocity at 440° in the absence of a catalyst. However, at 15° the rate of this reaction is too small to measure.

According to Ostwald, another important characteristic of catalysis is that extremely small amounts of the catalyst are able to bring about changes in large amounts of a given substance. This is an unfailing characteristic of all catalytic processes. We have already seen how minute quantities of colloidal platinum can act on large amounts of hydrogen peroxide. For the inversion of cane sugar, direct measurements have shown that this process (which also proceeds spontaneously at high temperatures) is catalyzed by 0.00000008 gram of H^+ in 1 cc. of solution¹ and the hydrogen ion concentration, which can be measured very accurately (p. 55), remains unchanged at the end of the reaction. In the same way, 0.0000001 g. $FeSO_4$ catalyzes the oxidation of potassium iodide² and 0.000000001 g. $CuSO_4$ catalyzes the oxidation of sodium sulphide.³ For other catalysts, values of the same order are effective.

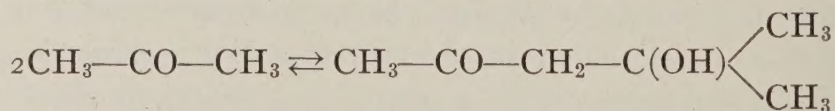
According to Ostwald, in all cases of true catalysis the catalyst remains wholly unchanged at the close of the reaction. Since it is very difficult to make direct analytical determinations of extremely small concentrations of the catalyst, it is easier to measure the constant of the reaction velocity and to make sure that the reaction remains of the first order (p. 24) in the presence of the catalyst. If this is so it is proof that the catalyst is not consumed in the course of the reaction.

In cases of autocatalysis, the amount of catalyst increases and a corresponding correction must be applied in determining the constant of the reaction velocity.⁴ For the complicated calculations in connection with this correction the special literature of the subject must be consulted.

The fundamental idea of Ostwald's conception is that the equilibrium of the reaction is not altered by catalysis. For a reaction between two substances, which yields two other products and does not go to completion, the action ceases whenever the velocity of the reaction between the two original substances is equal to the velocity of the reverse reaction between the resulting products, *i.e.*, when $v_1 = v_2$. Since $v_1 = k_1 C_1 C_2$ and $v_2 = k_2 C_3 C_4$ (pp. 25-26) this is true when

$$\frac{k_1}{k_2} = \frac{C_3 C_4}{C_1 C_2}$$

The experimental proof also confirms this conformity to law. For example, the reversible reaction of the condensation of acetone



under the influence of various catalysts and various initial concentrations of acetone, diacetone alcohol and the catalyst, comes to equilibrium when the ratio of the concentrations corresponds to the K in the formula for the law of mass action (p. 26).⁵ For some unstable catalysts, apparent exceptions to this

¹ Smith, W. A. *Zeitschr. f. physikal. Chem.* 25: 144. 1898.

² Mayer, O. *Chemiker-Zeitschr.* 27: 662. 1903.

³ Titow. *loc. cit.*

⁴ Philoche. *Jour. de chim. phys.* 6: 213 and 355. 1908.

⁵ Koelichen. *Zeitschr. f. physikal. Chem.* 33: 129. 1900.

rule have been observed but in such cases a secondary reaction can always be found, which changes the quantity of the given catalyst.¹

There is a very important conclusion from this. If the equilibrium of a reaction is expressed by the equation

$$\frac{k_1}{k_2} = \frac{C_3 C_4}{C_1 C_2} = K$$

the constant k_1 is increased in the presence of the catalyst but the equilibrium is not disturbed, *i.e.*, the value of $\frac{k_1}{k_2}$ is unchanged. This proves that the reverse

reaction is likewise accelerated; its constant k_2 increases so that $\frac{k_1}{k_2}$ has the same value as in the absence of the catalyst. *Therefore catalysts accelerate reversible reactions in both directions.* For example, if the decomposition of A, which forms the simpler substances B and C, is hastened by the effect of a certain catalyst, the same catalyst must, also, under suitable conditions, accelerate the reverse process of synthesis of A from B and C. Innumerable confirmations of this conclusion have been found in studies of the action of plant enzymes but it was the physico-chemical work on the kinetics of catalytic processes which encouraged experimental investigation toward the bold idea of bringing about synthetic enzyme action.

It should be remembered that the reversibility or, as it is often called, the "reversion of catalysis" correlates with the knowledge of thermochemistry. Berthelot's basic principle, which says that reactions proceed alone only when they involve the liberation of heat, is valid only for the cases in which much heat is developed. If there were no limit to the idea there could be practically no reversible reactions because each reaction proceeds with the development of heat in one direction but with an absorption of heat in the other direction. Endothermic reactions are actually often spontaneous and take on energy at the expense of cooling the system. Of course endothermic reactions are impossible at absolute zero, but at the so-called ordinary temperature (about 20°C.) only those proceed which involve but a slight consumption of energy. At higher temperatures various endothermic reactions take place spontaneously. All hydrolyses involve only a small release of heat. Hence the syntheses require so little energy that they are able to proceed without the application of heat. The same thing holds for all other reversible catalytic processes which take place at correspondingly low temperatures. However, in laboratory experiments with catalytic reactions which absorb energy it is advisable to raise the temperature if possible.

As for the theory of catalysis, it seems that there is no one explanation for all cases. Although in many other theoretical problems the various preliminary explanations have finally fused into a uniform general principle, this will be very difficult in the case of catalysis.

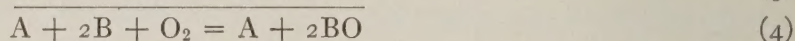
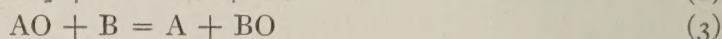
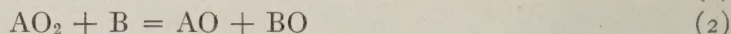
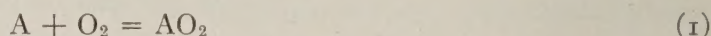
The older views of Liebig² and Nägeli,³ which supposed catalysts to have certain "molecular vibrations" which could disrupt the molecules of other substances, are now outgrown. These views were not based on experimental proof and are the more untenable because they lead to the confusion of catalytic processes with the phenomena of liberating energy. At present many cases of catalysis may be satisfactorily explained by the very widespread "theory

¹ Tammann. *Zeitschr. f. physikal. Chem.* 3: 25. 1889; 18: 426. 1895; 37: 257. 1901; Idem. *Zeitschr. f. physiol. Chem.* 16: 281. 1895; Medwedeff. *Pflügers Arch. f. ges. Physiol.* 74: 193. 1899; and many others.

² Liebig. *Poggend. Ann.* 48: 106. 1839.

³ Nägeli, C. v. *Theorie der Gärung.* P. 29. 1879.

of intermediate products.”¹ According to this theory, the catalyst reacts with the substance to be altered and the original reaction is so modified that its velocity increases. We have already seen that this explanation applies particularly to chemical induction. It is enlightening that this explanation covers the cases of catalysis which are undoubtedly connected with induction because the change from induction to catalysis is only a matter of changing the velocities of some coupled reactions. Consider the following coupled reactions of oxidation which have already been described:



If reaction 2 goes faster than 3 there is an induction but if reaction 3 has a higher rate than 2 the whole process is catalysis because in this case the sum of the partial reactions indicates a change according to equation 4 and the catalyst A is unmodified. The oxidation of powdered charcoal by metallic sodium at 300°C. is an example of such a reaction. Sodium peroxide Na_2O_2 is first formed and this oxidizes the charcoal much faster than molecular oxygen. The sodium is completely reduced and the process can be repeated. Thus a small amount of sodium can oxidize large quantities of charcoal.² Intermediate products are undoubtedly produced by a series of catalytic processes. There are two characteristics of such catalyses. In the first place, the temperature coefficient of such processes is normally one for chemical reactions though the reaction velocity increases two or three times for each 10° interval of temperature. Secondly, such catalysts are very specific in their action. Since the nature of the intermediate products limits the action to a certain substance, the catalyst is adjusted to its substrate by what E. Fischer calls the “lock and key” relationship.

Another theory of catalysis appears to be quite justified because it is the best explanation for many phenomena. According to this theory, the enzymes provide the most favorable medium for chemical reactions—a medium in which the catalyzed reaction proceeds faster than in the usual solution. For example, at 25° methyl acetate dissolved in benzene is scarcely saponified by triethylamine but if an emulsion is formed by shaking with 2% water, the drops of water act as catalysts. Since both ester and base dissolve in them, they react with each other more easily and the reaction is thereby accelerated.³ This is a simple model of such catalytic effects. It is known as “catalysis in heterogeneous systems” or briefly as “heterogeneous catalysis.” Of course, under this comes all cases in which adsorption of the reacting substances is very important. The concentration of the adsorbed substance increases at the surfaces and the reaction is hastened. Hence it must be borne in mind that many supposed enzymes probably do not exist in the sense of specific catalysts, and the apparent effects of enzymes in such cases are caused by various surface phenomena which are never lacking in a colloidal system (*cf.* p. 27). The activation of hydrogen by spongy platinum is an analogous effect because the hydrogen is apparently dissolved in the platinum in the true sense of the word.⁴

The cases just described belong in the category in which catalysis is due to the increase in the reaction velocity either in the phase which functions as a catalyst or at the surfaces (the latter type is called adsorption catalysis). Sometime the temperature quotient of heterogeneous catalysis does not correspond to that of a regular chemical process. Often it is less than 2, contrary to van't Hoff's rule (p. 25), and conforms to the value of the quotient of diffusion—a purely physical process. This is because in many cases the reaction velocity in the phase which holds the catalyst is larger than the rate of diffusion of the reacting substance

¹ Bredig. *Ergeb. d. Physiol.* for 1902. P. 177.

² Bamberger. *Ber. chem. Ges.* 31: 451. 1898.

³ Bredig. *Anorganische Fermente.* 1901. P. 92.

⁴ Mond, Ramsay and Shields. *Proc. Roy. Soc. London.* 62: 290. 1898; *Idem.* *Zeitschr. f. physikal. Chem.* 19: 25. 1896.

from another phase.¹ It is evident that the rate of diffusion then practically represents the reaction velocity of the catalyzed reaction. This easily explains the anomalous temperature coefficient. From the standpoint of the theory of intermediate products it would be extremely difficult to explain these temperature coefficients which are so often observed in catalytic processes.

In accord with the peculiar mechanism of heterogeneous catalysis, its specificity is much less marked than that of catalysis by intermediate products. All the effects of colloidal solutions of metals as described by Bredig are probably to be interpreted as heterogeneous catalysis.

The third theory of catalysis has been worked out by v. Euler.² The catalyst is thought to increase the ionization of the reacting substances, since it usually affects only reactions of ions and since the reactions of organic substances therefore proceed with measurable velocities because the electrolytic dissociation of these substances is so small. Euler's theory applies to the many cases of heterogeneous catalysis as well as to that by intermediate products. It is especially suited to catalytic hydrolyses. The real difficulty of this theory is to explain the complicated catalytic phenomena such as appear in the simultaneous action of two or more catalysts.³ All these phenomena are too little known as yet and they present difficulties to every theory. For the present, even the dependence of the acceleration of the reaction upon the amount of catalyst cannot be expressed by a valid relationship. In some cases we find a velocity which is proportional to the quantity of catalyst but in other cases such a proportionality⁴ is lacking. Experimental evidence to establish quantitative laws for these variations has not yet been produced. The variability may be illustrated by the 30% increase in the effect of acidity upon the hydrolysis of cane sugar when neutral salts are added.⁵

It is impossible to consider here the other theories of catalysis. It is sufficient to note that there are numerous theories which seek to connect catalysis with radiant energy.⁶

9. ENZYMES OF PLANTS

It has already been mentioned that the principal material transformations in organisms take place through the agency of chemical induction or catalysis. The organic catalysts of living cells are known as enzymes and are apparently substances of colloidal nature.^c

The problem of enzyme study is very interesting. Its importance is exceedingly great. The explanation of the details of the chemistry and modes of action of the enzymes is fundamental to an understanding of the material transformations which underlie the vital functions. So far we have not reached this goal because we do not even know the chemical nature of a single enzyme. As Bunge⁷ puts it, nobody has ever seen the enzymes. To be sure, there are preparations of various enzymes some of which have been introduced on a

¹ Nernst. *Zeitschr. f. physikal. Chem.* 47: 52. 1904.

² Euler, H. v. *Chemie der Enzyme*. 2nd Ed. 1920.

³ Schade, H. *Zeitschr. f. exp. Pathol. und Therapie*. 1: 603. 1905; Ipatiew. *Ber. Chem. Ges.* 45: 3205. 1912.

⁴ O'Sullivan and Thompson. *Jour. Chem. Soc. (London)* 57: 847. 1890; Kastle and Loevenhardt. *Amer. Chem. Jour.* 24: 491. 1900; Schütz, E. *Zeitschr. f. physikal. Chem.* 9: 577. 1887; 30: 1. 1900; Medwedeff. *Pflügers Arch. f. ges. Physiol.* 69: 249. 1896; 74: 193. 1899; 81: 540. 1900.

⁵ Arrhenius, S. *Zeitschr. f. physikal. Chem.* 4: 237. 1889.

⁶ Lambert. *Compt. rend.* 138: 196. 1904; Barendrecht, H. P. *Zeitschr. f. physikal. Chem.* 49: 456. 1904; 54: 367. 1905; Callow, Lewis and Nodder. *Jour. Chem. Soc. (London)* 109: 55 and 67. 1916; Lewis, M. C. *Ibid.* 109: 796. 1916; 111: 457. 1917; 113: 471. 1918; 115: 182, 710 and 1360. 1919.

^c It has been reported recently (Miller, E. R. *Science*. 70: 356-357. 1929) that a tyrosinase extracted from a mushroom with water was found to diffuse through a dialyzing sack of cellophane. This contradicts the conception of all enzymes as colloidal substances.—Ed.

⁷ Bunge. *Lehrbuch der physiol. Chem.* 4th Ed. 1898. P. 171.

commercial basis but all these preparations are mixtures of different substances including several enzymes in each. The latest studies of Willstätter and his colleagues have produced very pure preparations.^d

The slow progress in the chemical investigation of enzymes is due to the difficulties of isolating them in pure form. They are chiefly colloidal in nature and occur in the tissues in small amounts. There are still no chemical methods of isolating small quantities of colloids in pure form from a mixture of large amounts of other colloidal substances such as proteins, many carbohydrates and other components of protoplasm. In any case there is no basis for considering enzymes to be proteins as many writers assumed at one time. This led them to attribute a particular vital importance to the proteins in contrast with all the other components of living cells. It was a purely speculative bias which fortunately has no effect to-day.

Adsorption phenomena seem to play an important rôle in enzyme action since it is possible to obtain a high concentration of certain enzymes by adsorbing them to proteins, powdered lead phosphate or other substances¹ or even to filter paper. The latter method has long been used² to separate enzymes by virtue of their unequal capacity for adsorption (capillarization). The electrical properties of these enzymes and of the adsorbent are important in this connection. Thus, substances which readily take up basic dyes, adsorb but weakly certain enzymes which are closely adsorbed by other adsorbents which are easily colored by acid dyes (pages 4 and 10).³ Analogous phenomena, which bespeak the colloidal nature of enzymes, have appeared in the cataphoresis of these substances.⁴

The effect of increase in temperature correlates with the colloidal properties of enzymes. All the enzymes now known lose their catalytic action at 100°C. Hence boiling is a sure means of preventing enzymatic decomposition and syntheses in plant material. The catalytic action is usually destroyed even at 60°C. but the enzyme often regenerates after some time.^e In the anhydrous condition and, therefore, in the form of carefully dried powder or in pure glycerine, enzymes are able to resist a somewhat higher temperature.

So we do not know the chemical nature of enzymes, pure preparations of them have never been obtained [but see note *d* above], and we judge of their presence in cells only by their specific effect. For this purpose, extracts in

^d At least one enzyme has now been so highly purified and carefully analysed that it is accepted by many as absolute proof of the chemical nature of enzymes. In 1926 Sumner (Jour. Biol. Chem. 69: 435-441. 1926) isolated from the jack bean, *Canavalia ensiformis*, a preparation of urease which was extremely active. The enzyme was obtained in crystalline form and shown to be a globulin.—Ed.

¹ Brücke, E. Sitzungsber. d. Akad. Wien, Math.-naturw. Kl. 43: 601. 1861; Danilewski, A. Arch. f. path. Anat. und Physiol. 25: 279. 1862; Cohnheim. Ibid. 28: 241. 1863; Neumeister. Zeitschr. f. Biol. 30: 453. 1894; Peters, A. W. Jour. Biol. Chem. 5: 367. 1908; Willstätter and others, see p. 44.

² Grüss, J. Biol. und Capillaranalyse der Enzyme. 1912.

³ Dauwe, F. Hofmeisters Beitr. 6: 426. 1905; Hedin. Biochem. Jour. 2: 81 and 112. 1907; Michaelis und Ehrenreich. Biochem. Zeitschr. 10: 283. 1908; 12: 26. 1908; and others.

⁴ Henri, V. Biochem. Zeitschr. 16: 473. 1909; Michaelis, L. Ibid. 16: 81. 1909; 17: 231. 1909; 19: 181. 1909; Iscovesco, H. Ibid. 24: 53. 1909; Lebedew, A. v. Ibid. 26: 221. 1910; and others.

^e This suggests the presence of a zymogen or precursor to the enzyme. Many of the regenerating enzymes are associated with respiration and the respiratory chromogens are thought by some to make the regeneration possible. Cf. Bakh and Oparin (Trans. Karpov Inst. Chem. 4: 217-231. 1925) and Keilin (Proc. Roy. Soc. London (B) 104: 206-252. 1929).—Ed.

water or glycerine are taken from plants and the enzymes are precipitated from them by alcohol. By means of repeated extraction and precipitation it is often possible to increase the enzyme content of the precipitate. Moreover, there are several methods of killing protoplasm without destroying the enzymes. It is better to destroy the structure of the living cells by mechanical devices rather than by the use of chemical agents. A common method is to grind the material with sand and diatomaceous earth and to squeeze the macerated mass in a hydraulic press at 200–300 atmospheres pressure.¹ The pressed juice contains many active enzymes. The tissue is often dehydrated by suitable liquids,² by freezing³ or by careful drying at moderate temperatures. The latter method injures many enzymes, however. After the protoplasm is dead the catalytic action of the material on the reaction that is characteristic of the enzyme concerned is tested according to one of the methods described above.

A much used and convenient method of studying enzymatic processes is autolysis or self-digestion of the tissue—a method introduced by the celebrated French agricultural chemist, Boussingault.⁴ The parts of plants in small pieces are moistened and left at a temperature suitable for enzyme action (30°–60°C.). The addition of an antiseptic such as chloroform, toluol, xylol, etc. does not interfere with the work of the enzyme. The protoplasm quickly disintegrates but many enzymes remain very active. In many cases the intensity of the enzyme action after autolysis exceeds that in living protoplasm because some enzymes are formed in large amounts during the autolysis.⁵ In other cases autolysis cannot be used because the given enzymes are rapidly destroyed by the self-digestion. The material for the action of the enzyme during the autolysis comes from the organs which are being digested. Autolysis has proved to be a valuable method for studying enzyme action especially because the enzymes appear in such large amounts. The effect of each enzyme is usually easy to distinguish from that of the other enzymes and the various organic catalysts are thereby available for investigation. Since many important chemical reactions in organisms proceed catalytically, the number of enzymes in one cell can be very large. The simultaneous action of ten enzymes has already been demonstrated⁶ and this number will undoubtedly be increased in time.

The specificity of enzymes is usually more restricted than that of inorganic catalysts. It is least among the enzymes of oxidation and reduction.⁷ However, the narrow specificity of enzymes really manifests itself only with respect to stereoisomerism. They are considerably more sensitive to the spatial

¹ Buchner, E., H. Buchner und M. Hahn. *Zymasegärung*. 1903. P. 58.

² *loc. cit.* Pages 237 and 243.

³ Palladin, W. *Zeitschr. f. physiol. Chem.* 47: 27. 1906.

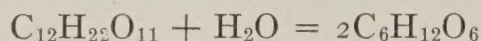
⁴ Boussingault, J. B. *Agronomie, chimie agricole et physiologie*. 6: 137. 1878.

⁵ Palladin und E. Popoff. *Biochem. Zeitschr.* 128: 487. 1922; Palladin und S. Mansky. *Ibid.* 135: 142. 1923; Euler, H. v. und R. Blix. *Zeitschr. f. physiol. Chem.* 105: 83. 1919; Euler und J. Laurin. *Ibid.* 106: 312. 1919; Euler und O. Svanberg. *Ibid.* 107: 269. 1919; Cf. also Oparin, A. und A. Bach. *Biochem. Zeitschr.* 148: 476. 1924.

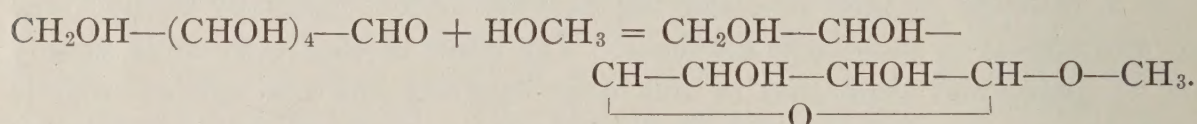
⁶ Dox. *Jour. Biol. Chem.* 6: 461. 1909; Idem. *Plant World*. 15: 40. 1912; Kammann. *Biochem. Zeitschr.* 46: 160. 1912; and others.

⁷ Bach, A. *Compt. rend.* 164: 248. 1917.

arrangements of atoms in the molecules than to the variations in the chemical composition of the substrate.¹ Different disaccharides, such as saccharose, lactose, maltose, etc., which have the same empirical formula $C_{12}H_{22}O_{11}$ and which decompose by hydrolysis according to the equation



can only be attacked by different specific enzymes.² No less specific is the action of the so-called proteolytic enzymes which effect the hydrolysis of proteins.³ Spatial isomerism is also involved here. The enzymes seem to be optically active bodies of asymmetrical structure. Hence each enzyme can only work on the asymmetrical substances of a certain structure. Actually, both the sugars referred to above and the proteins are optically active. Direct experience teaches us that polypeptides, the complexes formed of amino acids (the elementary units of structure of the protein molecules), are split by proteolytic enzymes only when they are composed of optically active amino acids. Indeed the amino acids concerned must show exactly the same direction of rotation of light that characterizes them in the bodies of plants or animals. If only one of the amino acids contained in a dipeptide fails to give the same rotation with which it occurs in natural products, the enzymes are unable to split this dipeptide.⁴ The glucosidases, *i.e.*, enzymes which hydrolyze the glucosides, furnish a beautiful illustration of the sensitivity of enzymes to the spatial arrangement of atoms and of their non-sensitivity to the unlike chemical composition of the substrate, except for the presence of atomic groupings which are not directly attacked by enzyme. Glucosides are acetal-like compounds of sugar with alcohols. Thus glucose and methyl alcohol form methyl glucoside:



Methyl glucoside is found in two configurations which are designated as α and β .⁵ This fact is explained by the occurrence of two glucoses which differ from each other in the angle of rotation of polarized light. The one which rotates it the most is called α -glucose and the other is β -glucose. So α -methyl glucoside rotates the plane strongly to the right but β -methyl glucoside produces a rotation to the left.⁶ Hence the two forms of methyl glucoside are represented by the following projection formulae:

¹ In order to avoid misunderstanding it must be kept in mind that the so-called "sensitivity" refers to the atomic groupings which are not altered by the enzyme.

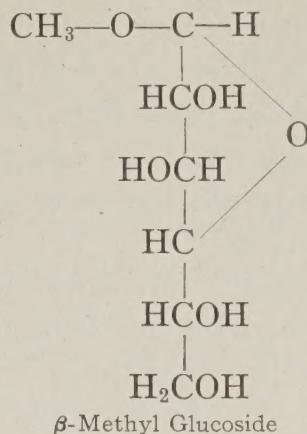
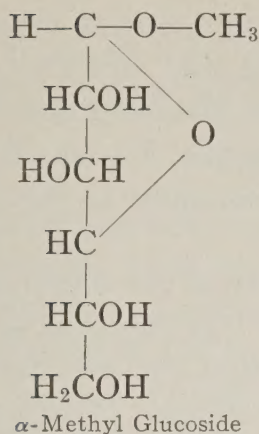
² Fischer, E. Ber. chem. Ges. 27: 2985. 1894; 28: 1429. 1895; Idem. Zeitschr. f. physiol. Chem. 26: 60. 1898.

³ Abderhalden, E. und A. Fodor. Zeitschr. f. physiol. Chem. 87: 220. 1913; Abderhalden und E. Schiff. Ibid. 87: 231. 1913; Abderhalden, G. Ewald und I. Watanaba. Ibid. 91: 96. 1914; and many others.

⁴ Fischer, E. und E. Abderhalden. Zeitschr. f. physiol. Chem. 46: 52. 1905; 51: 264. 1907.

⁵ Fischer, E. Ber. chem. Ges. 26: 2400 and 2478. 1893.

⁶ Eckenstein, A. v. Recueil des travaux chim. des Pays-Bas. 13: 183. 1894; Maquenne, L. Bull. Soc. Chim. de France (3) 3: 469. According to these investigators, the melting points and the solubilities of the α - and β -forms of glucose are also different.



It has been found that both kinds of glucosidases occur in plants.¹ Yeast extracts split chiefly α -glucosides and the extract of almond seeds splits chiefly the β -glucosides. However, it is to be noted that the enzyme from yeast splits different α -glucosides which differ greatly in the chemical structure of the alcohol portion.

Emulsin, the enzyme from almonds, for its part hydrolyzes all β -glucosides of the most varied chemical composition. For a long time an incorrect conception has prevailed concerning the properties of enzymes in relation to the substrate. The ability to attack only certain spatial isomers has been taken as a general proof of their very specific adjustment to the chemical nature of the substrate. This is incorrect. Enzymes show a specificity that is scarcely greater than that of inorganic catalysts. Since the latter are not asymmetrical substances they are passive in respect to the spatial isomerism of the substrate. However, on the basis of the foregoing erroneous conception, it is doubtful if the enzymes are to be regarded as true catalysts.² For the peculiar properties of enzymes, their thermolability and extremely specific action should be taken into consideration. Some workers also consider it hardly probable that enzymes work only by catalysis (*i.e.*, only by increasing the velocities of spontaneous reactions), since many enzymatic processes are not found outside of organisms and so do not start at all without the intervention of enzymes. Hence, Ostwald's definition of catalysis does not hold for the enzymes of living cells. According to this view, enzymes are to be regarded as the direct excitors of the material transformations which they control. For example, in this connection it would be claimed that glucose would undergo its various conversions under the influence of at least ten different enzymes. From the standpoint of true catalysis it would be assumed that in aqueous solution the sugar dissociates spontaneously into ten different kinds.

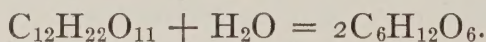
All these exceptions are still insufficiently based on facts. To all appearances, the thermolability of enzymes is associated with their colloidal nature and so has nothing to do with the characteristic nature of enzyme action. As for the specificity of enzymes, this is largely a matter of spatial isomerism. In

¹ Fischer, E. Ber. chem. Ges. 27: 2985. 1894; 28: 1429. 1895; Idem. Zeitschr. f. physiol. Chem. 26: 66. 1898.

² Bokorny, Th. Centralbl. f. Bakt., Parasitenk. und. Infektionskrankheit. (II) 21: 193. 1908; Loew, O. Ibid. 21: 198. 1908; Idem. Biochem. Zeitschr. 31: 159. 1911.

the presence of asymmetrical substances or in asymmetrical solvents some mineral catalysts are not passive in relation to spatial isomers.¹ One can scarcely doubt that there are actually less enzymes than are now estimated. In connection with the dissociation of the sugar molecule, it seems that glucose in a slightly alkaline solution can dissociate into not only 10 but about 100 different forms.²

Accordingly, the contradiction between the nature of the action of enzymes and that of inorganic catalysts is only superficial. On the contrary, various facts could be collected in favor of the assumption that enzymes act as typical catalysts. In the first place, a small amount of enzyme is able to control extremely large quantities of substrate. Invertase—the enzyme which hydrolyzes cane sugar—inverts 200,000 parts by weight of sugar for 1 part of enzyme preparation. Chymosin, which curdles the casein of milk, works in the ratio of 1 to 800,000. Determinations of the velocity constants of hydrolytic enzyme reactions also show that the enzyme is unaltered in the course of the process, *i.e.*, that the enzyme action corresponds exactly to Berzelius' conception of catalysis (p. 30). Thus the constant of the inversion of sugar under the influence of invertase is that of an equation of the first order;³ the reaction proceeds in the same way as it does when the inversion is induced by acids:



Hence the enzyme remains intact throughout the reaction. The same results have been obtained with catalase⁴ which decomposes hydrogen peroxide according to the equation $2\text{H}_2\text{O}_2 = 2\text{H}_2\text{O} + \text{O}_2$, and with erepsin, an enzyme which resolves polypeptides into amino acids.⁵ In some cases the constants fluctuate but this seems to be due to secondary effects, such as the destruction of the enzyme by side reactions, an increased adsorption of the enzyme, or an accumulation of the products of the reaction. Sometimes it is not improbable that chemical induction and not catalysis is involved.

The discovery of the reversibility of enzyme reactions is very significant. On the one hand it provides a conclusive proof that enzymes are nothing more than typical catalysts and, on the other hand, the possibility of enzymatic syntheses explains how the various organic substances are formed in cells. It has been shown that synthesis and hydrolysis can be controlled by a single enzyme.

According to the rule stated on page 33, enzymatic syntheses can be observed in reactions which involve a small liberation of heat. All hydrolyses come in this category and most of the enzymes known are hydrolyzing in their action.

¹ Bredig und Fajans. Ber. chem. Ges. 41: 752. 1908; Fajans. Zeitschr. f. physikal. Chem. 73: 25. 1910.

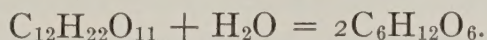
² Nef, J. U. Liebigs Ann. d. Chem. 403: 204. 1914.

³ O'Sullivan and Thompson. Jour. Chem. Soc. (London) 57: 834. 1890; Hudson. Jour. Amer. Chem. Soc. 30: 1160 and 1564. 1908; Taylor. Jour. Biol. Chem. 5: 405. 1909.

⁴ Euler, H. v. Hofmeisters Beitr. 7: 1. 1905; Waentig und Steche. Zeitschr. f. physiol. Chem. 76: 177. 1911.

⁵ Euler, H. v. Arkiv. för kemi. Vol. 2. 1907.

Synthetic action of enzymes was first demonstrated for maltase which hydrolyzes the disaccharide maltose into two molecules of glucose



The reaction involves a slight liberation of heat. On the basis of theoretical considerations, C. Hill¹ subjected a 40% solution of glucose to the action of a yeast preparation containing maltase for several months at 30°C. There was a marked change in the rotation of the solution and in the reducing power of the sugar. A disaccharide had been formed but it was not identical with maltose.² It was given the name "isomaltose" but an exact chemical identification was lacking. Later synthetic products were obtained with other enzymes which hydrolyze di- and polysaccharides. Lactase, which decomposes lactose to form equal amounts of galactose and glucose, in concentrated solutions of these hexose sugars gives rise to isolactose³—a disaccharide which has not been studied much. An unconfirmed statement has also been made that cane sugar can be synthesized from glucose and fructose by an enzyme which is not identical with invertase.⁴ In yeast juice there is an intensive hydrolysis of glycogen but if 30% sugar is added the result is a synthesis of this polysaccharide.⁵

Analogous phenomena have been observed for the fats. The enzyme lipase splits them into glycerine and free acids if sufficient water is present. With stronger concentrations of glycerine and acids and therefore in the presence of relatively less water, the reverse process of synthesis of fats sets in.⁶

Syntheses by enzymes have also been found in the third physiologically important group of substances—the proteins. The formation of precipitates and jellies in solutions of albumin after the addition of chymosin (which curdles milk) is held to be the result of synthesis of proteins and the products are known as plasteins.⁷ In this case it is impossible to test the accuracy of the assumption by exact chemical methods. The same difficulty enters into all analogous experiments which aim at the synthesis of large proteins from simpler substances,⁸ because the chemical nature of proteins is not yet known and so all experimentation lacks a sound foundation. However, an enzymatic formation

¹ Hill, C. Jour. Chem. Soc. (London) **73**: 634. 1898.

² Emmerling, O. Ber. chem. Ges. **34**: 600 and 2206. 1901; Hill, C. *Ibid.* **34**: 1380. 1901; Armstrong, E. F. Proc. Roy. Soc. London (B) **76**: 592. 1905; *Idem.* Proc. Chem. Soc. (London) **19**: 209. 1904.

³ Fischer, E. und E. F. Armstrong. Ber. chem. Ges. **35**: 3144. 1902.

⁴ Pantanelli, E. Atti d. Reale Accad. dei Lincei, rendiconti (5). **15**(1): 587. 1906; **16**(2): 419. 1907. On the basis of later discussions these statements are to be regarded as superseded (*überholt*).

⁵ Cremer, M. Ber. chem. Ges. **32**: 2062. 1899.

⁶ Hanriot. Compt. rend. **132**: 212. 1901; Loevenhardt. Amer. Jour. Physiol. **6**: 331. 1902; Kastle and Loevenhardt. Amer. Chem. Jour. **24**: 491. 1900; Pottevin. Compt. rend. **137**: 1152. 1903; *Idem.* Ann. Inst. Pasteur. **20**: 901. 1907; Dietz, W. Zeitschr. f. physiol. Chem. **52**: 279. 1907; Welter, A. Zeitschr. f. angew. Chem. **24**: 385. 1911; Iwanoff, S. L. Formation and transformation of fat in seed-plants. 1912. (Russian).

⁷ Danilewsky, Lawrow und Salaskin. Zeitschr. f. physiol. Chem. **36**: 277. 1902; Sawjaloff. Pflügers Arch. f. ges. Physiol. **85**: 171. 1901; Nürenberg. Hofmeisters Beitr. **4**: 543. 1903; Payer. *Ibid.* p. 554; Henriques und Gjaldbak. Zeitschr. f. physiol. Chem. **71**: 485. 1911; **81**: 439. 1912; Glagoleff. Biochem. Zeitschr. **50**: 182. 1913; **56**: 195. 1913; and others.

⁸ Robertson, T. B. Biochem. Jour. **3**: 95. 1907; *Idem.* Jour. Biol. Chem. **5**: 393. 1908; Taylor. *Ibid.* **3**: 87. 1907; Iwanoff, N. N. Transformations of the nitrogeous substances in yeast. 1919 (Russian); and others.

of amorphous protein-like substances from crystalline amino acids has probably been accomplished in recent times.¹

Long ago a whole series of facts showed that enzymatic syntheses of various substances take place in organisms but up until recently there were large gaps in this field of knowledge. In the first place, not a single synthetic product has been isolated in pure form and chemically identified. Secondly, the reversibility of enzymatic reactions had not been clearly proven. A number of facts suggested that enzymes could only form substances which they were unable to break down. For these reasons some workers were led to claim that there were specific enzymes for synthesis, distinct from the hydrolyzing enzymes.² However, such a distinction is hardly legitimate³ since the reversibility of enzyme action has been established beyond doubt. It appears that hydrolyzing enzymes function as true catalysts and do not even disturb the point of equilibrium of the reversible reactions.

The pioneer studies of Bourquelot and his colleagues⁴ solved this complicated question. These investigators have synthesized glucosides by enzymes under such conditions that the products have been obtained by the kilogram in pure crystalline form and there is no doubt of the reversibility of enzyme action. The earlier contradictory results of other workers are satisfactorily explained. At that time there were no pure preparations of enzymes and C. Hill and all his successors worked with a mixture of various enzymes. Hence they always obtained a mixture of different end-products. Bourquelot and his colleagues have overcome this difficulty by using for their syntheses the β -glucoside-splitting emulsin of almonds and the α -glucoside-splitting enzyme of yeast. Unlike most other enzymes, these remain active in alcoholic solution. Emulsin can even be used in 80–95% alcohol. So if glucose is dissolved in an excess of methyl or ethyl alcohol and emulsin is added to the solution, conditions are most favorable for a synthesis of glucoside. Since the amount of water is small in proportion to the total amounts of glucose and alcohol, the point of equilibrium is shifted in favor of synthesis and actually more than half the glucose is recovered in the form of the glucoside. On the other hand, on account of the higher percentage of alcohol only the emulsin is able to be very active. The other enzymes present might synthesize various disaccharides but their action is checked because the amount of alcohol is appreciably greater than that of sugar. The alcohol takes a predominant part in the reaction.

Thus the experimental conditions worked out by these writers give the glucosidase an advantage over all other enzymes and favor synthesis. As usual, nature answers clearly under the proper conditions but gives vague infor-

¹ Taylor, A. E. *Jour. Biol. Chem.* 3: 87. 1907; 5: 381. 1908; Wasteneys and Borsook. *Ibid.* 62: 15 and 633. 1924–25; 63: 563. 1925; but cf. Rona und Chrometzka. *Biochem. Zeitschr.* 189: 249. 1927.

² Euler, H. v. *Zeitschr. f. physiol. Chem.* 52: 146. 1907.

³ Barendrecht, H. P. *Biochem. Zeitschr.* 118: 254. 1921.

⁴ Of the numerous papers by these authors, only those are cited here which summarize the facts. Bourquelot et Bridel. *Jour. de phar. et de chim.* (7) 4: 385. 1911; 5: 388. 1912; 6: 1. 1912; 9: 104, 155 and 230. 1914; Bourquelot. *Ibid.* 8: 337. 1913; Bourquelot et Aubry. *Ibid.* 12: 15 and 182. 1915; 14: 65. 1915; 15: 246 and 273. 1917; Bourquelot, Bridel et Aubry. *Ibid.* 16: 353. 1917; Review up to 1914 by Bourquelot. *Ibid.* 10: 361 and 393. 1914.

mation if the conditions are otherwise. Bourquelot obtained glucosides of different alcohols in hundreds and thousands of grams and in pure crystalline form from pure glucose and alcohol through the action of enzymes. If the glucose is insoluble in an alcohol it can be dissolved in acetone or ethyl acetate. By these methods the workers have obtained many α - and β -glucosides and galactosides of various alcohols. Analogous methods have also given the disaccharides gentiobiose, galactobiose and others. These results remove all doubt of the possibility of enzymatic syntheses. However, still another important result has been achieved. If emulsin in alcoholic solution is allowed to act on a prepared glucoside or on a mixture of the two components of this glucoside (glucose and alcohol), the result in either case is an equilibrium at equal concentrations of glucose and glucoside. In a typical experiment emulsin in an 85% ethyl alcohol solution of ethyl glucoside and glucose changed them to equal molecular concentrations. In both cases equilibrium comes at 0.150% glucose in the solution. The same experiment in 60% alcohol gave the same point of equilibrium for hydrolysis and synthesis with 0.276% glucose. For methyl glucoside equilibrium in 80% methyl alcohol—with 0.054% glucose and 40% methyl alcohol—was reached at 0.194% glucose for both synthesis and hydrolysis. The same results were obtained with α -glucosides. Temperature has no effect on the equilibrium, as one would expect. A more detailed study of the kinetics of the glucoside enzyme has shown that the velocities of hydrolysis and synthesis are identical with equal concentrations of alcohol and sugar. The hydrolysis and synthesis of β -methyl glucoside in 30% methyl alcohol proceeded at the same rates and equilibrium was reached at the same time in both cases.

Time	Synthesis		Hydrolysis	
	Rotation of polarized light	Change in rotation	Rotation of polarized light	Change in rotation
0	+64'	0'	-42'	0'
1	+58'	6'	-36'	6'
2	+48'	16'	-26'	16'
4	+40'	24'	-18'	24'
8	+28'	36'	- 6'	36'
13	+16'	48'	+ 6'	48'
18	+12'	52'	+10'	52'
	(Equilibrium)		(Equilibrium)	

The results obtained by Bourquelot and his colleagues prove the reversibility of enzyme reactions. This is an important achievement in the field of enzyme studies. It gives us an idea of the mechanism of material transformations in living cells. For example, if there is a rapid formation of starch in the plastids we know that there is, there, a high concentration of sugar and not much water. When starch is being hydrolyzed there must be more water present. In the

cell sap, which is a very dilute aqueous solution of various substances, synthetic processes are improbable. In the protoplasm, however, there is a large amount of jelly-like colloids and proportionally less water; these conditions favor synthetic processes. The ease with which the physical state of the protoplasmic colloids can be changed is naturally of great significance for the velocity and the velocity constant of hydrolytic enzyme reactions in living cells.

In order to carry out enzymatic syntheses, the work should be done in sirupy and gelatinous media whenever possible and never in dilute aqueous solutions. It is still better to use organic liquids as solvents.¹ The temperature can be raised as long as the enzyme is uninjured by the high temperature. This is particularly liable to occur in alcohol and other organic solvents. Each enzyme has its specific optimum temperature at which it works faster than at higher and lower temperatures. Likewise there is an optimum pH for the action of each enzyme.

It should be noted that the investigations of Bourquelot and his colleagues not only provide proof of the identity of enzymatic processes with catalytic phenomena but also help to make catalysis clearer. The Ostwald theory stands for a fixed point of equilibrium in the case of catalyzed processes but up to the present this has not been proved for inorganic catalysts. Bourquelot furnished an unequivocal proof with biochemical material which was more suitable than inorganic substances for the purpose. Thus Ostwald's theory of catalysis has been placed on a firm basis for the first time.

Recently the study of enzymes has taken a new direction. Various experiments have been undertaken to make clear the chemical nature of enzymes. On the one hand, there are new methods for the isolation and purification of these substances: on the other hand, various methods have been employed to increase the enzyme content of tissues in order to facilitate the isolation of them.

The work of Willstätter and his colleagues² gave very pure preparations of enzymes. Selective adsorption was used to extract and purify the enzymes. As we have already noted, some substances adsorb chiefly small particles with a certain charge. For example, loam adsorbs acid substances and kaolin basic substances. Some enzymes behave like weak acids (lipase), others like weak bases (trypsin) while a third type are amphoteric (see p. 57). On the basis of these differences, it is possible to separate lipase, amylase and trypsin from pan-

¹ Willstätter and Stoll have made chlorophyll from its component parts—chlorophyllid and phytol—through the action of chlorophyllase on chlorophyllid in a solution of phytol. Under natural conditions the function of chlorophyllase is probably the synthesis of chlorophyll. In recent times there have been reports of various enzymes which remain active in dilute alcohol and other organic solvents.

² Willstätter und Stoll. *Liebigs Ann. d. Chem.* 416: 21. 1918; W. und Racke. *Ibid.* 425: I. 1921; 427: III. 1922; W., Oppenheimer und Steibelt. *Zeitschr. f. physiol. Chem.* 110: 232. 1920; W. und Steibelt. *Ibid.* 111: 157. 1920; W. und Kuhn. *Ibid.* 116: 53. 1921; W. und Csanyi. *Ibid.* 117: 172. 1921; W., Graser und Kuhn. *Ibid.* 123: I. 1922; W. und Wassermann. *Ibid.* 123: 181. 1922; W. und Waldschmidt-Leitz. *Ber. d. Deutsch. Chem. Ges.* 54: 2988. 1921; Willstätter. *Ibid.* 55: 3601. 1922. (A review of the preceding papers); W. und Schneider. *Zeitschr. f. physiol. Chem.* 133: 193. 1924; W. und Waldschmidt-Leitz. *Ibid.* 125: 132. 1923; 134: 161. 1924; W., Waldschmidt-Leitz und Hesse. *Ibid.* 126: 143. 1923; W., Schneider und Bamann. *Ibid.* 147: 248. 1925; W., Schneider und Wenzel. *Ibid.* 151: I. 1926; W. und Bamann. *Ibid.* 151: 242 and 273. 1926; 173: 17. 1928; W., Bamann und Waldschmidt-Graser. *Ibid.* 173: 155. 1928.

creatic juice as follows. Loam adsorbs all three of the unpurified enzymes but this adsorption is limited by the substances carried over with the enzymes. When the loam is discharged electrically, the acid lipase leaves the surface and goes into solution. The other less acid or even basic enzymes remain adsorbed. Repetition of this process effects a separation of the enzymes; then begins the purification. The invertin of yeast as adsorbed by loam contains about 65 to 75% impurities of which the greatest part are gums from the yeast. Continued purification increases the adsorptive power of the enzymes. Finally it exceeds that of the accompanying substances. Then the more or less pure invertin is adsorbed by kaolin while the gums remain in solution and can be removed. It is possible to purify this enzyme further by methods which cannot be used on the crude preparation. The chief difficulty in preparing enzymes lies in the necessity of isolating insignificant amounts of a colloid from a mixture with large amounts of a foreign colloid. The purification of invertin has been carried so far that the preparation does not contain a trace of proteins or carbohydrates and the concentration of the enzyme is 1600 times that in living yeast. From this it appears that invertin is free from phosphorus. Lipase and peroxidase can also be highly purified. The latter is reddish-brown in color while the purified preparations of other enzymes are colorless.

Willstätter¹ believes that active enzymes are heterogeneous systems. They consist of two components—the colloidal carrier and the active catalyst. Only the latter has a specific effect which it can exercise, however, only in conjunction with the carrier. The nature of the latter is not very important and the active substance can pass from one carrier to another but the strength of the enzyme action is thereby altered.

This new direction of enzyme investigation is very welcome. The science has too long been governed by another less welcome method of approach which confined itself either to theoretical matters or to studies which did little to solve the problem of enzymes. There are still many who work by this method.

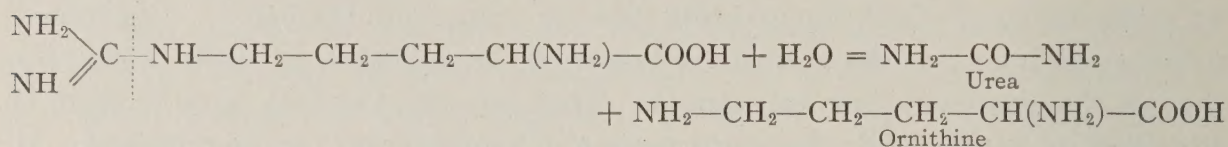
The general scheme is as follows. If a transformation within an organism can be shown to take place after the protoplasm is suitably killed, this chemical reaction is called an enzymatic process. In order to prove the statement, it is held to be sufficient to show that the reaction does not take place after the material is heated to 100°C. Many writers have sought to use this proof and the investigation of the chemical side of the enzymatic processes has been neglected. Only the latest work in biochemistry has emphasized this problem which is so important for physiology. It has been erroneously thought that proof of the enzymatic nature of a certain process was a satisfactory demonstration of the nature of the process and the interest of the investigator was thereby satisfied. Such standpoints are quite wrong because we have found that the catalyst merely changes the velocity of the chemical reaction and proof of catalysis tells nothing of the nature of the material transformation in question.

Moreover, this experimental method is unreliable and leads to serious errors. Above all it is not admissible to declare an action enzymatic because it stops after the materials are heated. In the presence of large amounts of colloids (which are found in every plant material) the reaction may cease after boiling for various reasons. Often the discovery of a new enzyme is claimed on the basis of such experiments. Such claims are not based on experimental proof but on the hasty assumption that each enzyme effects but one given reaction. Hence the nomenclature is flooded with countless names of newly discovered enzymes though the enzymes

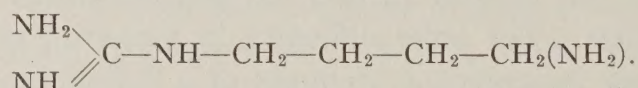
¹ Willstätter. Ber, chem. Ges. 55: 3601. 1922; W., Schneider und Wenzel. *loc. cit.*

many times do not exist in living cells but only in the imagination of their finders. We have already found that a specific action of enzymes is usually found only in relation to spatial isomerism.

Under these circumstances, it is necessary to provide a new method of identifying enzymes. Actually certain constant features of enzyme action can be used for their identification. The latest work shows that enzymes unite with their substrate. Kiesel¹ showed that the enzyme arginase which splits arginine to urea and ornithine



does not act on decarboxylated arginine—the so-called agmatine



This is remarkable because the molecule is split at a point which does not adjoin the carboxyl group. Edlbacher and Bonem² then proved that even the methyl ester of arginine is not split by arginase. The dipeptide prepared from two molecules of arginine (p. 278) yields only half the theoretical amount of urea, probably because one of the two carboxyl groups disappears when the dipeptide is formed. On the basis of this it may be supposed that arginase unites with the carboxyl group of arginine.

Kuhn³ carried out an analogous investigation with emulsin—the enzyme which splits glucosides. He showed that emulsin combines with the hydroxyl groups of the glucoside. Glucosides in which all the hydroxyl groups are fixed are not split by emulsion.

Even earlier Michaelis and Menten⁴ developed the view that an enzyme splits only substances with which it forms labile compounds. These writers even calculated the dissociation constants of some compounds of this kind.

This is the first constant which can be measured experimentally. Another constant of enzymes is the velocity constant of the cleavage of different substances. Josephsohn⁵ proved that different glucosides are split by enzymes in an order different from that by means of acids. For example, salicin is harder to split by acids than arbutin: the velocity constants are 117 for salicin and 300 for arbutin. On the contrary, salicin is more easily split by the enzyme than arbutin. In this case the constants are 160 for salicin and 14 for arbutin. The dissociation constants of glucosidase for various glucosides are as follows:

Methyl glucoside.....	1.4
Salicin.....	33
Helicin.....	62
Arbutin.....	24

Probably still other constants of enzyme action will be calculated and a physical-chemical identification of enzymes is therefore possible. Perhaps the number of enzymes will be reduced as this work is perfected.

Some investigators have assumed that enzymes are split off from the substances which are then worked upon by the enzymes. According to this view, the chemical nature of enzymes is similar to that of the substrate. It has been claimed that various carbohydrates split off diastase—the enzyme which hydrolyzes starch—through the action of gaseous HCl followed by ammonia.⁶ It would follow that proteolytic enzymes are only mixtures of certain amino

¹ Kiesel, A. *Zeitschr. f. physiol. Chem.* 118: 284. 1922.

² Edlbacher, S. und P. Bonem. *Ibid.* 145: 69. 1925.

³ Kuhn, R. *Ibid.* 143: 154. 1925; 135: 1. 1923.

⁴ Michaelis, L. und M. L. Menten. *Biochem. Zeitschr.* 49: 333. 1913.

⁵ Josephson, K. *Zeitschr. f. physiol. Chem.* 147: 1. 1925.

⁶ Panzer, Th. *Ibid.* 93: 316 and 339. 1914; 94: 10. 1915.

acids.¹ The statement was later made² that diastase is obtained from starch by the action of boiled saliva or even the ash of saliva. On the other hand, the idea has been developed that aldehydes (*e.g.*, formaldehyde) act like diastase on starch under certain conditions.³ The same simple schemes have been proposed to explain the action of oxidizing and reducing enzymes.⁴ All these experiments contradict numerous critical observations and experiments⁵ and the solution of the problem cannot yet be arrived at. Conceptions of the nature of enzymes are changing fast⁶ and important discoveries in the field of enzyme investigation will soon come. Their importance for the theoretical science as well as for technical applications will be immeasurable.

The question of the regulation of enzyme action will be treated in another book in connection with other problems of regulation and coördination. We need to consider only the regulatory principles and the governing factors.

Recently some investigators, in consideration of the very promising results of enzyme studies, have expressed the belief that investigation of enzyme action alone can solve all problems of material transformations in living cells.⁷ Such views are to be regarded as unfounded at present. If a mixture is made of all the enzymes to be found in a cell, the sum total of their action does not resemble in the least the biochemical processes which go on in the protoplasm. Aside from enzymes, the mechanical structure of living cells is necessary for the continuance of vital processes. It follows that biochemical reactions do not run by chance but by coördination.

The work of Palladin⁸ and his colleagues shows very plainly that if the protoplasm is killed by mechanical action, or in other words, if the internal structure of the cell is destroyed the enzymes begin to work like the separate parts of a disrupted chemical system with all the substances mixed freely.

In killed cells many enzymes are destroyed by other enzymes. Moreover, the enzymes are not kept apart from the storage products and accordingly substances are formed in killed cells which could never appear by normal physiological processes.

In conclusion we must consider factors which serve to regulate and coördinate enzyme action. Substances which retard the work of enzymes are known as anti-enzymes.⁹ Their nature is even more obscure than that of the enzymes themselves. It is probable that their action is often connected with surface

¹ Herzfeld. *Biochem. Zeitschr.* 68: 402. 1915.

² Biedermann. *Fermentforschung.* 1: 385 and 474. 1916; 2: 458. 1917; 3: 70. 1919; 4: 458. 1919; 8: 395. 1919; 9: 122. 1919; Haehn, H. und H. Berentzen. *Chemie d. Zelle und Gewebe.* 12: 286. 1925.

³ Woker, Gertrude. *Ber. chem. Ges.* 49: 2311. 1916; 50: 679. 1917; 51: 790. 1918; *Biochem. Zeitschr.* 99: 307. 1919; Maggi, H. *Fermentforschung.* 2: 304. 1919.

⁴ Begemann, O. H. K. *Pflügers Arch. f. ges. Physiol.* 161: 45. 1915.

⁵ Wohlgemuth. *Biochem. Zeitschr.* 94: 213. 1919; Jacobi, Kaufmann, Levite und Sallinger. *Ber. chem. Ges.* 53: 681. 1920; Sallinger. *Fermentforschung.* 2: 449. 1919; Schulz. *Ibid.* 3: 72. 1919; and others.

⁶ Cf. *e.g.*, Palladin und Popoff. *Biochem. Zeitschr.* 128: 487. 1922; Palladin und Mansky. *Ibid.* 135: 142. 1923.

⁷ Cf. *e.g.*, Höber. *Physikalische Chemie der Zelle und Gewebe.* 2nd. Ed. 1906. P. 364.

⁸ Palladin, W. *Die Arbeit der Fermente in lebenden und getöteten Pflanzen.* 1910; *Idem.* *Fortschritte der naturwissenschaftlichen Forschung*, Bd. I. 1910; Petruschewsky, A. *Zeitschr. f. physiol. Chem.* 50: 251. 1907; Korsakowa, M. *Ber. bot. Ges.* 28: 334. 1910; and many others.

⁹ Czapek, F. *Ber. bot. Ges.* 20: 464. 1902; 21: 4. 1903; Hedin, S. G. *Biochem. Jour.* 1: 474 and 483. 1906; Jacobi. *Biochem. Zeitschr.* 34: 485. 1911.

phenomena and may be due to the adsorption of enzymes which thereby become inactive "zymogens."¹ Artificial zymogens of urease have been prepared recently² by the use of the metals nickel, cobalt, copper and especially zinc. Urease can be regenerated from these zymogens. Other enzymes require the use of different methods for the preparation of artificial zymogens. In the living cells, zymogens are changed to active enzymes by means of the so-called kinases, the nature and method of action of which have been very little studied. The so-called co-enzymes are quite different from the kinases and they do not activate the zymogens. They rather stimulate the work of the already active enzymes.³

The idea of a co-enzyme is not clearly defined. In one sense it is often used for the medium suited to the enzymatic process. For example, a slightly acid medium favors the action of most enzymes though some require an alkaline reaction. Chymosin is active only in the presence of calcium salts. Many other co-enzymes are substances of apparently complex composition. The effect is perhaps often an indirect one.⁴ It must be borne in mind that statements concerning the substances which accompany enzymes are extremely defective.⁵ Kluyver and Struyk dispute the existence of the co-enzyme of alcoholic fermentation—the so-called co-zymase.⁶

In conclusion it should be pointed out that some enzymes are found only within cells and are known as intracellular enzymes. The so-called extracellular enzymes, on the contrary, are secreted by the cells and effect their transformations in the surrounding medium.

10. CLASSIFICATION AND CHARACTERISTICS OF PLANT ENZYMES⁷

Hydrolyzing Enzymes.—To this group belong the great majority of all known enzymes. The rational nomenclature is as follows: An enzyme is named according to the substance which it splits (or synthesizes) with the addition of the suffix -ase. Some enzymes which have been known a long time have other names by which they are correctly known in addition to the rational name.

¹ Vines, S. *Ann. Bot.* Vol. 11. 1897; Frankfurt, S. *Landw. Versuchsst.* 47: 449. 1897; Green, F. *Ann. Bot.* 7: 121. 1893; and others.

² Jacobi, M. *Biochem. Zeitschr.* 104: 316. 1921; 128: 80. 1922; Jacobi und Shimizu. *Ibid.* 128: 89, 95 and 100. 1922.

³ Detmer, W. *Zeitschr. f. physiol. Chem.* 7: 1. 1882; Hérissé. *Compt. rend.* 143: 49. 1901; Starkenstein. *Biochem. Zeitschr.* 24: 210. 1910. [The recent work of Doby and Hibbard (*Jour. Biol. Chem.* 73: 405-416. 1927) with sugar beets in sand cultures indicates that certain ions in the usual nutrient solutions may activate plant enzymes. They report that the chloride ion activates amylase while saccharase is stimulated by the nitrate ion.]

⁴ Harden and Young. *Jour. of Physiol.* Vol. 22. 1904; *Proc. Roy. Soc. London (B)* 77: 405. 1906; 78: 369. 1906; 80: 299. 1908; 81: 336. 1909; 82: 321. 1910; Abderhalden und Wertheimer. *Fermentforschung.* 6: 1. 1922.

⁵ Palladin believes that the term "zymogen" needs revision. Cf. Palladin und Mansky. *loc. cit.*

⁶ Kluyver, A. J. und A. P. Struyk. *Proc. Kon. Akad. Wetensch. Amsterdam.* Vol. 30, No. 5. 1927; *Biochem. Zeitschr.* 201: 202. 1928. So far only co-zymase has been mentioned. The disproof of the existence of co-zymase really means a disproof of the term "co-enzyme."

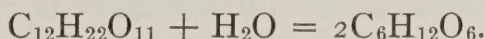
⁷ This is only a short review of plant enzymes to give a preliminary orientation. Further information will be given with the description of the several material transformations in plants. For the special literature of enzymes see: Euler, H. v. *Chemie der Enzyme.* 2nd Ed. 1920; Oppenheimer. *Die Fermente und ihre Wirkungen.* 4th Ed. 1913; [Waksman and Davison. *Enzymes.* 1926; Waldschmidt-Leitz. *Enzymes* (translated by Walton) 1929].

Enzymes Which Hydrolyze Carbohydrates.—*Diastase* or *amylase* is an enzyme which hydrolyzes starch to the disaccharide maltose. This was the first enzyme to be discovered and studied.¹

The quantitative relations between enzyme and substrate were first worked out for diastase. The optimum temperature is 50°–55°C. and the reaction must be slightly acid. *Cytase*, an enzyme which hydrolyzes cellulose, is just as widely distributed as diastase. *Pectinase* is an enzyme which hydrolyzes the middle lamella of cell walls. *Inulase* splits inulin, a polysaccharide much like starch but differing in yielding fructose instead of glucose when completely hydrolyzed. *Glycogenase* splits glycogen, another polysaccharide.

Trisaccharides are hydrolyzed to disaccharides and free hexoses by specific enzymes. Here belong *melicitase* and *raffinase* which hydrolyze melizitose and raffinose. The resulting disaccharides are broken down further by other specific enzymes.

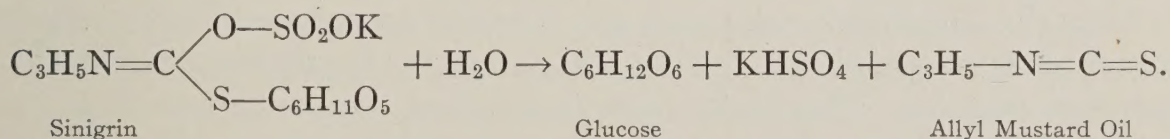
The specific action of all these enzymes manifests itself in that each disaccharide can be broken down only by its own enzyme although all of them are hydrolyzed according to the simple equation



Even when the cleavage products of two stereoisomeric disaccharides are identical, they can be split only by two different enzymes.

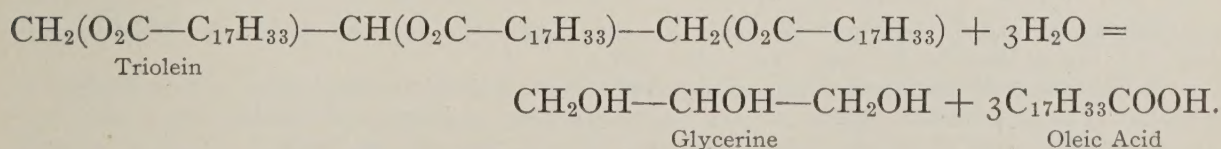
Invertin (invertase) or *saccharase* splits cane sugar into glucose and fructose. It also attacks α -fructosides although β -fructosides, glucosides and galactosides are not affected by this enzyme. Invertase is very common in plants and the kinetics of its action are well known. *Trehalase*, *maltase* and *cellase* hydrolyze trehalose, maltose and cellobiose into two molecules of α -glucose. *Lactase* splits lactose (milk sugar^f) into glucose and galactose.

Emulsin or β -glucosidase splits all β -glucosides. α -Glucosidase is different and splits the α -glucosides. It has already been mentioned that we have here an especially interesting case of specific enzyme action. Aside from these glucosidases, others have been described as splitting glucosides of peculiar structure. In the *Cruciferae* we find the enzyme *myrosin* which hydrolyzes sinigrin according to the equation



The physiological significance of this process is unknown.

The Enzymes Which Hydrolyze Fats are known as *lipases*. They split fats into glycerine and free fatty acids. Thus triolein is hydrolyzed as follows:



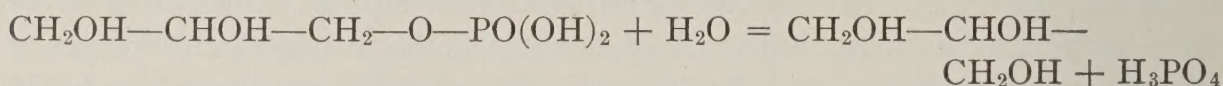
¹ Payen et Persoz. Ann. de chim. et de physique (2) 53: 73. 1833.

^f Lactose is not found in plant tissues.

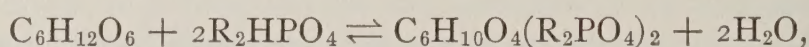
The individuality of the several lipases is not easily established because it is hard to separate the various fats.

The esters of various acids are hydrolyzed by the so-called *esterases* which are scarcely different from the lipases. The following are examples of this type.

Glycerophosphatase is an enzyme which splits glycerophosphoric acid, an important constituent of lecithin, into glycerine and phosphoric acid:

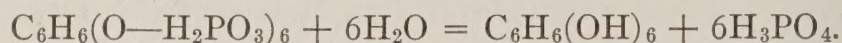


Hexosephosphatase hydrolyzes hexose diphosphate which seems to play an important rôle in the alcoholic fermentation of sugars. It is supposed that this enzyme effects the synthesis as well as the hydrolysis of hexose phosphate. This is shown by the equation

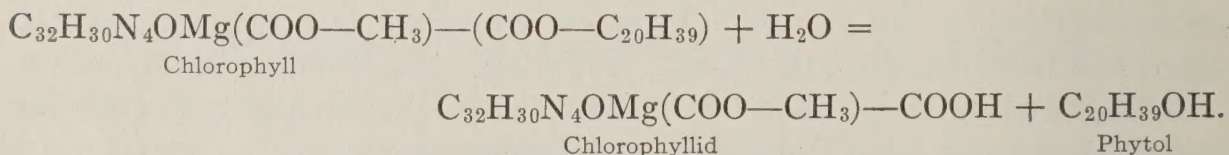


in case hexose diphosphate is formed.

Sulphatase, an enzyme recently found in the so-called taka yeast,¹ hydrolyzes compounds of ethyl sulphuric acid. *Phytase* splits phytin or inosit-phosphoric acid according to the equation



Chlorophyllase is found in leaves. It splits chlorophyll into chlorophyllid and phytol. The same enzyme regenerates chlorophyll from these components.



The enzymes which hydrolyze proteins are divided into four groups:

1. *Proteases*, which split proteins to peptones²
2. *Ereptases* or *Peptases*, which decompose peptones into crystalline amino acids
3. *Tryptases*, which remove amino acids from protein molecules³
4. *Deaminases* and *Deamidases*, which decompose the amino acids.

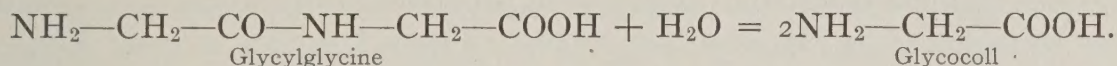
Since the individuality and even the elementary structure of many proteins has not yet been established, it is not always easy to identify the enzymes which hydrolyze proteins. This is the more pronounced because protein hydrolysis usually proceeds very rapidly and the stage of forming amino acids is reached quickly. Special methods are often used to discover the intermediate products in the formation of peptones. The presence of proteases has been proved with certainty for germinating seeds and fruits and particularly in the insec-

¹ Kurono, K. und C. Neuberg. Biochem. Zeitschr. 140: 295. 1923; Neuberg und Linhardt. Ibid. 142: 191. 1923; Noguchi, J. Ibid. 144: 138. 1924.

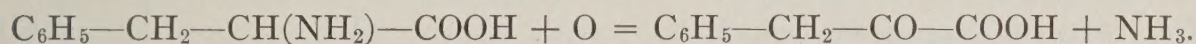
² The idea has arisen lately that the peptization of proteins is not hydrolysis but a change in the disperse condition of the cell colloids. Hence it would be a purely physical process. Accordingly, proteases would not be hydrolyzing enzymes and not even enzymes at all. On this point see Peroff, S. Mitt. d. milchwirtschaftlichen Instituts in Wologda. 2: 163. 1921; also Inichow. Biochem. Zeitschr. 131: 47. 1922.

³ Some investigators deny the existence of these enzymes.

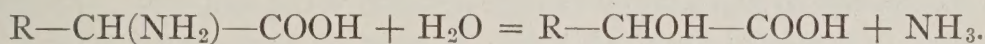
tivorous plants¹ where they appear as extracellular enzymes. Peptases and tryptases are very commonly found. The majority of these enzymes require a weak acid reaction but some work better in a slightly alkaline solution. The nature of the action of peptase may be seen in the following hydrolysis of a dipeptide:



According to the latest ideas,² deamidases must be sharply distinguished from the deaminases. The former deal only with the amide group CONH_2 while the latter are associated with the amine grouping CHNH_2 . Deaminization often takes the form of an oxidation process as *e.g.*

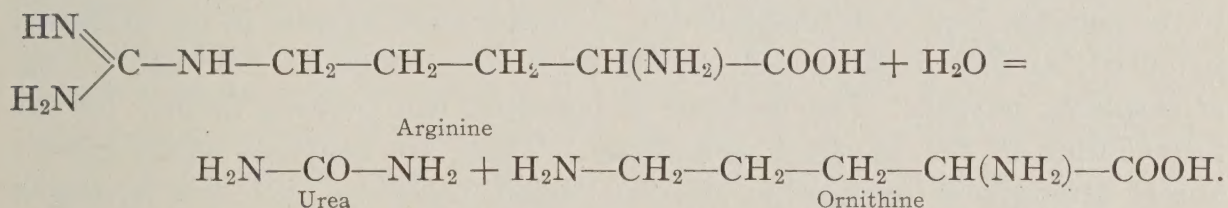


The oxidizing enzymes take part in such a process.³ The deaminases, in the true sense of the word, effect only hydrolytic cleavages:

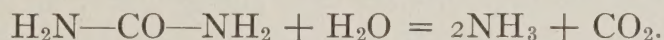


However, it usually happens that there is an oxidation in intermediate stages of the reaction.

The enzyme *arginase* can also be added to the deaminases. It splits urea from the molecule of arginine according to the equation



This reaction may be regarded as a possible method of the formation of urea in plants as well as in animals. Urea itself is decomposed by the enzyme *urease* which qualifies for the group of deamidases:



Enzymes which coagulate proteins are very common in plants. They have been found in the fruits of the paupaw tree (*Carica papaya*), in the digestive glands of insectivorous plants and in many other seed-plants and fungi. The physiological significance of these enzymes has not been accurately determined. It is not improbable that they play an active rôle in the important alterations of the disperse conditions of the protoplasmic colloids.

The *nucleases* hydrolyze nucleic acids to form purine and pyrimidine bases. The nucleic acids are a constituent of the stable proteins of the nucleus and are therefore one of the building materials of the cell structure. Hence it is not improbable that nucleases in cells effect not only hydrolyses but also syntheses.

¹ Vines. Jour. Linnean Soc. 15: 427. 1877; Idem. Ann. Bot. 11: 563. 1897; 12: 545. 1898; 15: 563. 1901; Goebel. Pflanzenbiol. Schilderungen. 2: 186. 1893; Abderhalden und Teruuchi. Zeitschr. f. physiol. Chem. 49: 1. 1906; and others.

² Butkewitsch, W. Anniv. vol. for K. A. Timiriaseff. 1916. P. 457.

³ Oparin, A. Biochem. Zeitschr. 124: 90. 1921.

This supposition is supported by the fact that nucleases are particularly common in germinating seeds and in young, growing organs.

The Oxidizing and Reducing Enzymes.^g Perhaps these substances are not always to be regarded as true enzymes, since they are made to include labile peroxides of a high oxidation potential and chemical compounds which take part in various coupled reactions. Thus some processes of oxidation and reduction are to be regarded as chemical inductions. This is also true of most inorganic catalysts of oxidation. The mechanism of all these reactions has been fully investigated and will be described in Chap. VIII which deals with respiration and oxidation processes in plants.

Unstable substances of the peroxide type are called *oxygenases*. Their chemical nature is still unknown although they seem to be organic substances. The reduced oxygenases, which have split off the labile oxygen, are called *oxidases*. They absorb oxygen and again become oxygenases. In many cases this absorption of oxygen can be determined directly by gas analysis. It can often be recognized by the sudden change in color of the material. For example, when a slice is removed from an apple, a pear or some other fruits, the cut surface at once becomes brownish because the oxidases, which are separated from the atmospheric oxygen in the intact fruit, absorb oxygen rapidly and change color.

It is enlightening that the change of oxygen from the molecular condition to the peroxide form is accompanied by a considerable increase in the oxidation potential, so that oxidases can oxidize substances which do not take on the atmospheric oxygen. The increase in potential can proceed further by the intervention of the so-called *peroxidases*. These are enzymes which cannot absorb atmospheric oxygen but activate various peroxides, including hydrogen peroxide. For example, the latter works extremely slowly on hydroquinone and other divalent phenols; but oxidation occurs at once when peroxidase is added.

The oxidizing enzymes of plants act very energetically on polyphenols. The first oxidizing enzyme to be studied was *laccase* which comes from the Japanese lacquer tree *Rhus vernicifera*,¹ the sap of which darkens in the air and becomes an excellent lacquer. Hence the formation of lacquer is an oxidation process. There is a very interesting preparation of laccase from *Medicago sativa* which can be purified and shown to be a mixture of calcium salts of some oxy-acids.² From this it is evident that the oxidizing enzymes of plants do not need to be organic derivatives of manganese or iron, although the salts of these two metals stimulate the action of oxidizing enzymes and are found in many enzyme preparations.³

^g For a complete treatment of these enzymes see **Kastle, J. H.** The Oxidases. U. S. Hygienic Lab Bull. No. 59. 1910.—*Ed.*

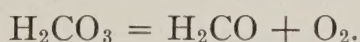
¹ **Yoshida.** Jour. Chem. Soc. (London) 43: 472. 1883.

² **Euler, H. v. und Bolin.** Zeitschr. f. physiol. Chem. 61: 1. 1909.

³ **Bertrand, G.** Ann. de chim. et de physique (7) 12: 115. 1897; **Idem.** Compt. rend. 124: 1032 and 1355. 1897; **Idem.** Bull. Soc. Chim. de France (3) 17: 619 and 753. 1897; *cf.* also **Trillat, A.** Compt. rend. 137: 922. 1903; 138: 94 and 274. 1904; **Idem.** Bull. Soc. Chim. de France. 31: 807. 1904; **Livache.** Compt. rend. 124: 1520. 1897.

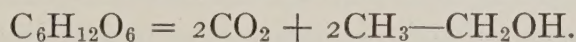
Tyrosinase, an enzyme which changes tyrosine to various dark-colored products of simultaneous oxidation and polymerization,¹ occurs in different mushrooms and roots of seed-plants. It is a widely distributed substance. *Oenoxidase* is found in various juicy fruits. It appears to oxidize the tannins and causes the browning of fruit juices exposed to the air. *Alcohol-oxidase* of the acetic acid bacteria probably activates hydrogen rather than oxygen since the process of acetic acid fermentation will also go on in the absence of oxygen under suitable conditions. This will be discussed further in Chap. VIII.

The peroxidase of horse-radish roots is useful for experiments in enzymatic oxidation, because it can be prepared in a very pure condition by means of new methods of preparation.² The reducing enzymes or *reductases* are very similar to the oxidizing enzymes in mode of action. Processes of reduction play an important rôle in the life of plants and the value of the reduction potential is often surprising. The biochemical reaction which is important not only for plants but for the entire organic world—the synthesis of organic substance from carbon dioxide and water—is nothing but a grand process of reduction which involves an enormous increase in chemical potential:

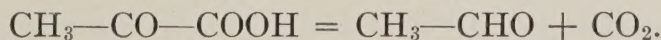


The assimilation of nitrate nitrogen by plants is decidedly a process of reduction because neither proteins nor nitrogen-containing constituents of protoplasm contain oxidized nitrogen. Fermentations are also combinations of intramolecular oxidations and reductions. All agents of fermentation have at their disposal large amounts of reducing enzymes and, under certain conditions, reduce substances which actually play no part in the physiological metabolism. For example, yeast is able to decolorize methylene blue and to reduce molecular sulphur to hydrogen sulphide. Neither of these reactions could be due to a removal of oxygen because they contain no oxygen. In these cases it is necessary to add hydrogen.

The Enzymes of Fermentation. The hydrolyzing, oxidizing and reducing enzymes are substances of unknown chemical composition but they effect transformations which are known and which could not take place by purely chemical agencies without the intervention of the enzymes. However, there have also been discovered in plants enzymes which theoretically do not always bring about recognizable reactions. Such an enzyme is *zymase* which causes alcoholic fermentation—the cleavage of sugar into carbon dioxide and ethyl alcohol:³



Among the enzymes which take part in alcoholic fermentation, there is said to be *carboxylase* which removes CO_2 from α -ketonic acids, forming the corresponding aldehyde without adding hydrogen.



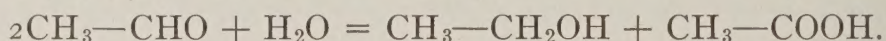
¹ Bourquelot et Bertrand. Jour. de Phar. et de Chim. (6) 3: 177. 1896; Idem. Bull. Soc. Mycol. for 1896. Pp. 18 and 27; Abderhalden und Guggenheim. Zeitschr. f. physiol. Chem. 54: 331. 1908; 57: 329. 1908.

² Willstätter and others. See page 44.

³ Buchner, E. Ber. chem. Ges. 30: 117 and 1110. 1897; 31: 568. 1898; Buchner, E., H. Buchner und M. Hahn. Die Zymasegärung. 1903.

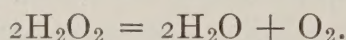
Carboxylase is also found in plants which do not bring about alcoholic fermentation.

Other enzymes also seem to take part in the complicated process of fermentation. For example, we know that in fermenting organisms there is an oxido-reductase which changes aldehydes to equimolecular amounts of alcohol and acid (the Cannizzaro reaction); *e.g.*



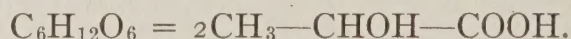
Such an enzyme has also been found in animal cells and named *mutase*. In the plant kingdom the Cannizzaro reaction has been established only in the case of yeast.¹

Catalases is the name given to the enzymes which remove molecular oxygen from hydrogen peroxide:



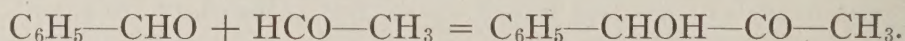
Some investigators consider catalases to be oxidizing enzymes while others think they reduce, but actually, they do neither. Yeast cells always contain considerable amounts of catalase yet it does not appear that it takes any part in the process of fermentation. Molecular oxygen is liberated chiefly during the assimilation of CO_2 by photosynthesis but catalases are found among the lower plants as well as in seed-plants. Of course the cleavage of hydrogen peroxide may be only a secondary effect of various kinds of enzymes and the independent existence of catalases is not entirely proved.

Lactolase, an enzyme supposedly present in the lactic acid bacteria, is said to make hexoses into lactic acid:

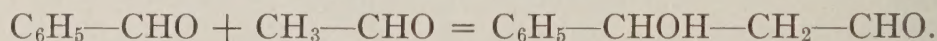


It is uncertain to what extent specific enzymes take part in this process (*cf.* Chap. VIII).

Carboligase is a supposed enzyme which is said to link aldehydes in a way that is not identical with aldol condensation:²



The aldol condensation proceeds as follows:



The existence of carboligase is poorly established. The latest critical studies show that the existence of enzymes of fermentation is uncertain. In some cases very active yeast cells and bacteria have been discovered in alleged solutions and preparations of enzymes.³ In other cases the fermentation is inoculated several times⁴ or stimulated by small amounts of poison;⁵ after filtration

¹ Kostytschew, S. Zeitschr. f. physiol. Chem. 89: 367. 1914; 92: 402. 1914.

² Neuberg und Hirsch. Biochem. Zeitschr. 115: 282. 1921; Neuberg und Liebermann. *Ibid.* 121: 311. 1921; Neuberg und Ohle. *Ibid.* 127: 327. 1922; 128: 610. 1922.

³ Kostytschew und Faermann. Zeitschr. f. physiol. Chem. 176: 46. 1928; Kostytschew und O. Schulgina. *Ibid.* 182: 50. 1929.

⁴ Kostytschew und A. Chomitsch. *Ibid.* 176: 55. 1928.

⁵ Kostytschew and V. Berg. in press.

through a bacterial filter, the action of the so-called enzymes of fermentation ceases but the action of hydrolyzing enzymes is not decreased at all by such treatment.¹ If it should finally be proved that there can be no cell-free fermentation under aseptic conditions, it will probably be necessary to find new terms for the cell constituents which have a certain organization and are not quite identical with the simple enzymes. Only such substances could be regarded as causes of fermentation.

In conclusion, the extraordinarily powerful catalytic action of enzymes is to be noted particularly. It surpasses by far the effect of inorganic catalysts. Proteins are usually hydrolyzed to amino acids by persistent heating in the presence of mineral acids. To carry the process to completion, 50 grams of protein must be boiled in a reflux condenser for at least six hours with fuming hydrochloric acid or sixteen hours with half-concentrated sulphuric acid. This is the strongest hydrolyzing effect which a chemist commands. The same hydrolysis of protein takes place in living plants by means of a tiny amount of proteolytic enzyme and at the temperature of the surrounding air. Polysaccharides, such as starch, can also be hydrolyzed by long heating with dilute mineral acids but in plant cells it takes place with an enormous velocity at the temperature of the air about the plant.

II. HYDROGEN ION CONCENTRATION AND ITS PHYSIOLOGICAL SIGNIFICANCE

We have found that the results of the dissociation of water play a leading rôle in various important biochemical processes but the so-called hydrogen and hydroxyl ions also control the reaction of the medium. This, in turn, influences the action of all the enzymes as well as the disperse condition of the protoplasmic colloids. This amounts to a dominant rôle in all the transformations within living cells. Hence in all biochemical studies it is very important that the reaction of the juices of plants be considered and accurately measured.^h

Ever since it has been known that an acid reaction depends on the hydrogen ions and an alkaline reaction on the hydroxyl ions, a merely *qualitative* determination of the reaction of various solutions or a rough estimate ("weakly acid," "strongly alkaline," etc.) has been considered unsatisfactory for most physiological questions. Hence in modern studies the reaction of the solution is measured *quantitatively* by direct measurements of the hydrogen or hydroxyl ion concentration.

Strong acids are substances which are strongly dissociated to form H ions. Likewise, strong alkalis are substances which split off a large amount of OH ions. On the contrary, weak acids and bases are but little dissociated at the same concentrations.² A solution of 1 mol of HCl in 32 l. of water is 97% dissociated into H^+ and Cl^- . The same solution of acetic acid is only 2.4% dissociated. Hence the so-called strength or weakness of acids and bases is determined by the value of its dissociation constant.

¹ Kostytschew and S. Klupt. In press.

^h For a complete account of our knowledge of the hydrogen ion concentration of plant tissues see the new monograph by Small entitled "Hydrogen-ion Concentration in Plant Cells and Tissues." 1929.—Ed.

² It must be borne in mind that only *molar* concentrations are considered here.

At infinite dilution all acids and bases are totally dissociated and thus equally strong but otherwise the degree of dissociation of either an acid or a base depends on the concentration. The product of the concentrations of ions divided by the concentration of the undissociated molecules is a constant, or

$$\frac{\alpha}{v} \cdot \frac{\alpha}{v} : \frac{1 - \alpha}{v} = \frac{\alpha^2}{v(1 - \alpha)} = k,$$

where $\frac{\alpha}{v}$ is the concentration of anions and cations, $\frac{1 - \alpha}{v}$ is the concentration of the undissociated molecules and v is the volume of the solvent in liters. The foregoing equation illustrates the so-called "dilution law" of W. Ostwald¹ which holds only for weak acids and bases. The dissociation constant for strong acids can be approximately measured by the relative rate of inversion of cane sugar. The constants for various acids are given in the following table.²

Hydrochloric acid.....	1.0000	Carbonic acid.....	4.4×10^{-7}
Nitric acid.....	1.0000	Hydrogen sulphide.....	5.7×10^{-8}
Sulphuric acid.....	0.5360	Boric acid.....	5.8×10^{-10}
Monochlor-acetic acid.....	0.0484	Hydrocyanic acid.....	4.7×10^{-10}
Formic acid.....	0.0154	Carbolic acid.....	6.0×10^{-11}
Acetic acid.....	0.0040	Glucose.....	6.6×10^{-13}

The differences between the dissociation constants of the strong and the weak acids are so great that at first glance it is not entirely conceivable that weak as well as strong acids in dilute solution require the same amount of alkali [in titration]. However, neutralization consists in the combination of H^+ and OH^- into undissociated molecules. For strong acids water is formed at the expense of the H ions already in solution. With weak acids new H ions are split off from the molecules of acid as fast as the consumption of the dissolved ions disturbs the equilibrium between ions and undissociated molecules. In this way, weak acids must use up just as much hydroxide as strong acids for equal strengths of solution.

The electrolytic dissociation of a weak acid or base can be regulated by the addition of salts of the same acid or base. Let us assume that we have added to 0.1 N acetic acid enough potassium nitrate to give a 0.1 N solution of potassium acetate in the solution of acid. Before the salt was added the solution contained 98.7% undissociated molecules of acid. The equation

$$\frac{(H^+)(CH_3COO^-)}{CH_3COOH} = k$$

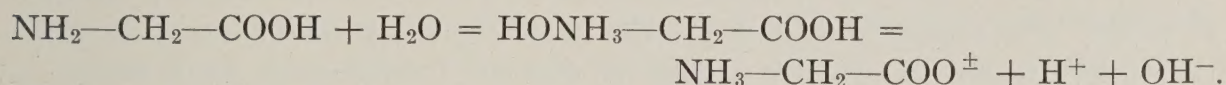
indicates the conditions of equilibrium where $k = 0.004$. But potassium acetate is strongly dissociated into K^+ and CH_3COO^- . After this salt was added the value of the denominator was scarcely changed while the value of

¹ Ostwald, W. *Zeitschr. f. physikal. Chem.* 2: 36. 1888; Rudolphi. *Ibid.* 17: 385. 1895; van't Hoff. *Ibid.* 18: 300. 1895.

² Nernst. *Theoretische Chemie*. 7th Ed. 1913. P. 583; Michaelis und Rona. *Biochem. Zeitschr.* 49: 232. 1913; 67: 182. 1914; and others.

CH_3COO^- was greatly increased. Since k cannot change there must be a corresponding decrease of H^+ . This lowers the acidity of the solution. In this way, any desired concentration of hydrogen ions can be obtained by mixtures of an acid and its salt. This is actually a means of regulating the hydrogen ion concentration in living cells.¹

Another means of preventing an injuriously high acidity or alkalinity in protoplasm is the so-called amphoteric electrolytes or ampholytes. These include substances which are able to split off both H^+ and OH^- and thus function as both acids and bases.² In this class we find some metallic hydroxides such as $\text{Al}(\text{OH})_3$ and $\text{Sn}(\text{OH})_2$. Of physiological importance we have the amino acids, purine derivatives, lecithins and proteins which furnish H^+ , OH^- and the "hemaphrodite ion" $\text{R}^\pm$. Thus glycocoll dissociates as follows:



It should be noted, however, that the basic and acid values of a given ampholyte are not equal. This condition is of secondary importance since it has meaning only in purely aqueous solution of salts of the ampholyte. In the presence of strong bases the formation of H^+ is increased in the amphoteric electrolyte as in general in a weak acid through the disturbance of the equilibrium (see above) as the result of neutralization, and the ampholyte is finally able to fix large quantities of strong bases. In an acid solution, however, the dissociation of the H ions is decreased and the ampholyte acts as a weak base. It has been mentioned already that strong and weak acids or bases have equal powers of neutralization. Hence a strongly acid or alkaline reaction of the solution is impossible in the presence of an excess of an amphoteric electrolyte. A simple experiment to test this is to take a solution of amino acids or of protein and to it add large amounts of strong bases or acids. It will be found that the reaction of the solution does not change much. After all the ampholyte has been used up in neutralizing the acid or base, there is a sharp change in the reaction if more is added.

The following table gives the basic and the acid dissociation constants of various amphoteric electrolytes.

Substance	$K_{\text{H}}(25^\circ)$	$K_{\text{OH}}(25^\circ)$
Glycocoll.....	1.8×10^{-10}	2.7×10^{-12}
Alanine.....	1.9×10^{-10}	5.1×10^{-12}
Leucine.....	1.8×10^{-10}	2.3×10^{-12}
Tyrosine.....	4.0×10^{-9}	2.6×10^{-12}
Phenylalanine.....	2.5×10^{-9}	1.3×10^{-12}
Aspartic acid.....	15.0×10^{-5}	1.2×10^{-12}
Histidine.....	2.2×10^{-9}	5.7×10^{-9}
Arginine.....	1.0×10^{-14}	1.0×10^{-7}
Lysine.....	1.0×10^{-11}	1.0×10^{-7}

¹ Henderson, L. J. and Black. Amer. Jour. Physiol. 21: 420. 1908; Henderson and Spiro. Biochem. Zeitschr. 15: 105. 1908.

² Bredig. Zeitschr. f. Electrochem. 6: 33. 1899; cf. also Winkelblech. Zeitschr. f. physikal. Chem. 36: 546. 1901; Walker. Ibid. 49: 82. 1904; Lunden. Ibid. 54: 532. 1906; Kanitz. Zeitschr. f. physiol. Chem. 47: 476. 1906.

Polypeptides and proteins give numbers of the same order. Solutions in which a strongly acid or alkaline reaction is prevented through the presence of such substances are called "buffered solutions."

In aqueous solutions it is impossible to have no hydrogen or hydroxyl ions because water itself is always slightly dissociated and so acts as an ampholyte. Hence the neutral reaction is nothing but a point at which the concentration of hydrogen ions is just equal to that of the hydroxyl ions. In pure water the concentration of H ions and OH ions is equal to $0.85 \times 10^{-7} = 10^{-7.07}$. The dissociation constant of water is $(0.85 \times 10^{-7})^2 = 0.72 \times 10^{-14} = 10^{-14.14}$. From this it is evident that all acid and alkaline reactions can be expressed in terms of the concentration of the hydrogen ions because the corresponding concentration of hydroxyl ions can be calculated from the dissociation constant of water. For example, if the concentration of hydrogen ions is equal to 10^{-13} , the concentration of OH ions equals 0.72×10^{-14} divided by $10^{-13} = 0.72 \times 10^{-1}$.

Sørensen¹ proposed that the measure of the hydrogen ion concentration be the negative logarithm of the corresponding concrete number, and that this should be spoken of as the pH. For example, if the concentration of hydrogen ions is $10^{-7.5}$ it is written: pH = 7.5. Hence the reaction of the liquid is acid if pH is smaller than 7.07 and alkaline if the pH is greater than 7.07.¹

Change of pH plays an important rôle in various physiological processes, such as the stimulation or retardation of enzyme action, the electrical discharge of cell colloids, the permeability of the plasma membrane, etc. Each plant can exist only within a certain range of pH.² Accordingly, many investigators are now engaged in measuring the pH of various biochemical processes.

12. METHODS OF DETERMINING PH

1. *Determination of pH from the Inversion of Cane Sugar.*—The inversion of cane sugar is a catalytic reaction in which the rate is directly proportional to the amount of catalyst, *i.e.*, of hydrogen ions. So the rate of inversion is measured simultaneously in two solutions, for one of which the pH is known exactly and the other being the solution to be studied. Then the pH of the unknown solution is calculated from the ratio of the two reaction velocities. This method is recommended for the determination of the affinity constants of various acids because it gives very exact results. The method is useless for physiological studies because the presence of neutral salts introduces large experimental errors.³

2. *Determination of pH from the rate of saponification of di-azo ethyl acetate* depends on the same principle. In this case the reaction velocity can easily be estimated from the volume of nitrogen liberated.⁴

¹ Sørensen. *Biochem. Zeitschr.* 21: 131. 1909.

² In ordinary usage the decimal fraction is omitted and neutrality is taken as pH 7.—*Ed.*

³ Arrhenius, O. *J. Gen. Physiol.* 5: 81. 1922; *Soil Science.* 14: 223. 1922; *Bodenreaction und Pflanzenleben.* 1922; Olsen, C. *Compt. rend. trav. Lab. Carlsberg.* 15: 1, 1923; Kurz, H. *Bot. Gaz.* 76: 1. 1923.

⁴ Arrhenius, S. *Zeitschr. f. physikal. Chem.* 4: 237. 1889.

⁵ Fränkel. *Zeitschr. f. physikal. Chem.* 60: 202. 1907; Fresenius, L. R. *Ibid.* 80: 481. 1912.

3. *Measurement of pH with the gas chain* is an extremely exact method, involves no sources of error and can be used for all kinds of solutions. The principle of the method¹ is the measurement of the electromotive force which results from the potential difference between the "hydrogen electrode" and the hydrogen ions in the solution that is being measured. Between a metal and dissolved ions of the same metal there is always a potential difference which disappears only when the solution is saturated with the metallic ions. Hence the size of the potential difference depends on the concentration of the ions in solution. To measure the pH one uses either the platinum electrodes saturated with hydrogen or the calomel electrodes.² Such "gas electrodes" are used like metallic ones and make it possible to determine the concentration of hydrogen ions because the amount of platinum passing into solution is so small that it need not be considered. Here too, the potential difference depends on the pH.

The actual measurement is made as follows. The electromotive force of a calibrated standard cell is first compared with that of a storage battery and then with that of the solution to be tested. When the latter is known the pH is calculated according to the Nernst equation:³

$$\text{pH} = \frac{\pi - 0.3380}{0.0577 - 0.0002(t^\circ - 18^\circ)} \quad (\text{for N/10 calomel electrodes})$$

where π is the electromotive force of the solution.

4. *Measurement of pH with Indicators*.—The first experiments to obtain a series of indicators which would correspond to different pH values were carried out in Nernst's laboratory.³ The principle involved is the change in color of each indicator at a certain pH. A series of suitable buffered mixtures of electrolytes are arranged to give a series of pH values. Each mixture is carefully calibrated by the gas chain method. Then a series of indicators are tested to discover those which change color at the various steps of pH. The indicator method is very simple to use but until recently its use was limited to solutions containing small amounts of salts or ampholytes. Clark and Lubs⁴ were the first to develop a series of indicators (derivatives of phenolsulphonphthalein) which are insensitive to ampholytes and salts and which have extraordinarily sharp color values. Through the increased use of these indicators the method has come into general use.^k

¹ Nernst. *Zeitschr. f. physikal. Chem.* 2: 613. 1888; 4: 129. 1889; Bugarszky und Liebermann. *Pflügers Arch. f. ges. Physiol.* 72: 51. 1898; Sørensen. *Ergeb. d. Physiol.* 12: 393. 1912; Michaelis, L. *Die Wasserstoffionenkonzentration*. 2nd Ed. 1922; Clark, W. M. *The Determination of Hydrogen Ions*. 2nd Ed. 1922, [or 3rd Ed. 1928].

² The latest development is the quinhydrone solution in which the platinized electrodes are saturated with hydrogen without passing hydrogen through it. Of course the method is limited to solutions in which the quinhydrone has no effect which is at pH < 7.8.

³ These calculations are eliminated for the user of the gas chain and potentiometer through the tables which are provided with each set of apparatus. The readings in volts are easily changed to the corresponding pH values.—Ed.

⁴ Salessky. *Zeitschr. f. Electrochem.* 10: 205. 1904; Fels. *Ibid.* 10: 208. 1904.

^k Clark and Lubs. *Jour. Wash. Acad. Sci.* 5: 609. 1915; 6: 481. 1916; Idem. *Jour. of Bact.* 2: 109 and 191. 1917; Clark, W. M. *The Determination of Hydrogen Ions*. 2nd Ed. 1922, [or 3rd Ed. 1928].

^k Manufacturers of scientific apparatus have developed several useful types of apparatus for the use of these indicators. Most of them provide for the comparison of the unknown solution plus a few drops of indicator with the standardized solution containing the same indicator. A recent form (the Hellige Comparator) uses colored glass discs in place of the standard solutions.—Ed.

CLARK AND LUBS' TABLE OF INDICATORS

Indicator	Conc. in %	pH		Color change	
		From	To ¹		
1. Thymol Blue (Thymolsulphonphthalein).	0.04	1.2	2.8	red	yellow
2. Brom Phenol Blue (Tetrabromphenol-sulphonphthalein).....	0.04	3.0	4.6	yellow	blue
3. Methyl Red (Orthocarboxybenzoazodimethylanilin).....	0.02	4.4	6.0	red	yellow
4. Brom Cresol Purple (Dibromorthocresol-sulphonphthalein).....	0.04	5.2	6.8	yellow	purplish-red
5. Brom Thymol Blue (Dibromthymolsulphonphthalein).....	0.04	6.0	7.6	yellow	blue
6. Phenol Red (Phenolsulphonphthalein)...	0.02	6.8	8.4	yellow	red
7. Cresol Red (Orthocresolsulphonphthalein).....	0.02	7.2	8.8	yellow	red
1a. Thymol Blue.....	0.04	8.0	9.6	yellow	blue
8. Cresolphthalein (Orthocresolphthalein)...	0.02	8.2	9.8	colorless	red

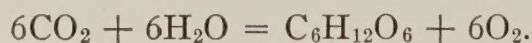
¹ Within these limits the pH is measured every 0.2 units by comparing the color values with those of calibrated buffered electrolyte mixtures. For the preparation of these mixtures see the original papers by Clark and Lubs [or Clark's book already mentioned].

It is interesting that the colorimetric method has been recently introduced into microchemical practice. It is also possible to measure the pH in the cell sap of living cells.¹ For this purpose it is necessary to use indicators to which the protoplasm is permeable. The ones commonly used are neutral red and methyl red. A series of buffered standard solutions is also required for these measurements.

13. THE INTERMEDIATE STAGES OF BIOCHEMICAL PROCESSES

The achievements of physical chemistry described on the preceding pages have clarified various biochemical processes which were once but vaguely understood. It is still more important that these physico-chemical principles have made it possible to attack physiological problems which otherwise could not be analyzed.

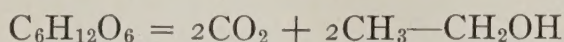
However, not all the difficulties have been met yet. Some material transformations are not understood because there are great gaps in the organic chemistry. That is to say, organic chemistry has no processes analogous to certain important biochemical reactions. Thus the photochemical synthesis of carbohydrates is governed by the mysterious reaction



The synthesis of amino acids from ammonia and sugar takes place constantly in plants but we cannot even write a summarized equation for this complex reaction. Alcoholic fermentation serves as a source of energy for a whole

¹ Rohde, K. Pflügers Arch. f. ges. Physiol. 138: 411. 1917.

series of microorganisms and is a fundamental process in various industries. This process goes on according to the summarized equation



but this apparently simple reaction can be neither artificially induced nor chemically understood. Analogous cases can be found in large numbers.

In recent times the problem of the chemical analysis of these vague biochemical processes has been attacked and appropriate methods of study worked out. Biochemical investigation in this direction will probably give results in the near future.

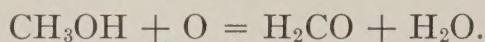
Most physiological reactions appear to be incapable of analysis because they are each a coördinated combination of several coupled reactions. For example, it is thought that the synthesis of carbohydrates in the light consists of the following processes:

1. $\text{CO}_2 + \text{H}_2\text{O} = \text{H}_2\text{CO}_3$
2. $\text{H}_2\text{CO}_3 = \text{H}_2\text{CO} + \text{O}_2$
3. $2\text{H}_2\text{CO}$ (Formaldehyde) $= \text{CH}_2\text{OH—CHO}$ (Glycolic aldehyde)
4. $\text{CH}_2\text{OH—CHO} + \text{H}_2\text{CO} = \text{CH}_2\text{OH—CHOH—CHO}$ (Glyceric aldehyde)
5. $2\text{CH}_2\text{OH—CHOH—CHO} = \text{CH}_2\text{OH—(CHOH)}_4\text{—CHO}$ (Glucose)
6. $2\text{CH}_2\text{OH—(CHOH)}_4\text{—CHO} = \text{C}_{12}\text{H}_{22}\text{O}_{11}$ (Maltose) $+ \text{H}_2\text{O}$
7. $n(\text{C}_{12}\text{H}_{22}\text{O}_{11}) = (\text{C}_6\text{H}_{10}\text{O}_5)_{2n}$ (Starch) $+ n\text{H}_2\text{O}$ etc.

In this scheme, formaldehyde, glycolic aldehyde and glyceric aldehyde play the rôles of so-called intermediate products. As long as the entire process proceeds smoothly these substances are at once carried further in the process and so do not accumulate. However, it is most important to isolate these intermediate products because the determination of their chemical nature clarifies the whole process. In the form in which the foregoing series of connected equations is written, the photochemical synthesis of carbohydrates in green plants is no longer mysterious because each of the equations can be understood. An experimental proof of this scheme has not yet been obtained but it serves to indicate the fundamental methods of investigation. The clarification of the chemistry of complicated physiological reactions requires the isolation of the intermediate products and analyses of their chemical natures.

It would seem at first glance that this problem could be solved by working with very large amounts of material. Also with the best possible coördination of the single chemical processes, the green leaves should still contain traces of unchanged formaldehyde and other intermediate products. From several kilograms of leaves it might be possible to obtain a couple of centigrams of formaldehyde. But this crude method may lead to entirely erroneous conclusions since, in addition to increase in the yield of intermediate products, various sources of error creep in. For example, in the case before us formaldehyde can be formed as a product of changes within the mixture. Chlorophyll, the green pigment of leaves, is an ester which includes methyl alcohol among its component parts. In working with a very large quantity of leaves there

might be a partial oxidation of the chlorophyll. This would tend to form formaldehyde from methyl alcohol:



Other methods of procedure must be found. In general the best way to obtain an accumulation of intermediate products is to check their further use in the process. This is the basic principle used in the isolation of intermediate products and the analysis of the chemistry of complicated biochemical reactions. By means of appropriate interference, we must try to disturb the normal coördination of the process to be studied in order that the intermediate products may not be used up so fast.¹ There are various methods available, the choice depending on the experimental conditions. For example, in working with living plants one can reduce the velocities of the processes which require considerable energy by cutting off the supply of oxygen or by reducing the supply of materials used in the operation. Then the intermediate products will gradually accumulate.² Very good results are also obtained by a direct seizure of the intermediate products so that they cannot be carried further in the reaction. Thus, aldehydes can be fixed with bisulphite,³ or oxidized to carboxylic acids and caught in the form of salts which are relatively insoluble in water but which dissolve in ether and can be washed out of aqueous solutions by the use of ether. Of course, some of these methods can be used only with killed plant material in case the latter contains active enzymes as it often happens. In such experiments satisfactory results can often be obtained by not going after the products themselves but by inactivating the enzymes which carry the given intermediate product to another stage in the process.⁴ For example, if in the normal course of events the primary intermediate product is oxidized or reduced, this product can be obtained by checking the action of the enzymes of oxidation or reduction. In such studies with living plants, the so-called time factor is very important. It is often possible to obtain the intermediate product only by running the experiment for a certain length of time. Too long or too short a time results in failure to isolate the intermediate product even with other conditions favorable.

In all such investigations it must always be borne in mind that the active intermediate products are generally unsaturated radicals with free valences which, of course, cannot be captured as such. The supposed intermediate products which are caught are generally secondary products and are stable transformation products of the foregoing unsaturated substances. It is evident

¹ On this point cf. Kostytschew. Ber. chem. Ges. 45: 1289. 1912; Idem. Zeitschr. f. physiol. Chem. 79: 130. 1912; 83: 93 and 105. 1913; 85: 493. 1913; Idem. Biochem. Zeitschr. 64: 237. 1914; Kostytschew und S. Zubkova. Zeitschr. f. physiol. Chem. 111: 132. 1920; Idem. Jour. d. Russ. Bot. Ges. 1: 47. 1916; 3: 40. 1918 (Russian); Connstein und Lüdecke. Ber. chem. Ges. 52: 1385. 1919; Neuberg und Hirsch. Biochem. Zeitschr. 100: 304. 1919; Neuberg, Hirsch und Reinfurth. Ibid. 105: 307. 1920; and many others.

² Kostytschew und Tswetkova. Zeitschr. f. physiol. Chem. 111: 171. 1920; Kostytschew und Afanassjewa. Jahrb. wiss. Bot. 60: 28. 1921; Idem. Tageb. d. I Kongr. d. russ. Bot. P. 87. 1921 (Russian).

³ Connstein und Lüdecke. loc. cit.; Neuberg. loc. cit.; Eliasberg, P. Jour. d. landwirt. Inst. in Petersburg. P. 74. 1921 (Russian).

⁴ Kostytschew. loc. cit.

from this that the chemical nature of processes can be determined only roughly by means of the identification of captured substances.

In some cases, positive results have been obtained by these methods. Hence it is not improbable that the exposition of all the chemical transformations in living plant cells is only a question of time. This possibility of direct determination of the chemical nature of important physiological processes has led to increased experimental work in the field of chemical plant physiology during recent years.

The question arises whether the solution of these biochemical problems [of intermediate products] will mean that the goal of chemical physiology is reached. This will certainly not be the case. All the work toward clarifying the chemical nature of physiological processes belongs in the field of pure chemistry. Physiology in the true sense of the word is concerned with the functions of the organism as a working apparatus. Now it is necessary to carry out the biochemical work for the problems of physiology but this is only preparation for the actual investigation of vital processes. For example, let us assume that the chemistry of alcoholic fermentation is satisfactorily explained. At once there arises the new question: How is the chemical energy changed into other forms of energy? Since in technical operations such a transformation of energy takes place by means of special apparatus it may be assumed that there is analogous apparatus in living cells. An investigation of this apparatus and its action will then be the next task in the problem of alcoholic fermentation. This is the real problem of physiology.

In this way experimental investigation is always going more deeply into the great problems of physiology. Consideration of the perpetual progress of our special branch of science leads us to hope that the most distant goal of physiology will eventually be reached.

CHAPTER II

THE ASSIMILATION OF SOLAR ENERGY BY GREEN PLANTS AND THE PRIMARY SYNTHESIS OF ORGANIC SUBSTANCES

1. NUTRITION THROUGH LIGHT WAVES AND ITS COSMIC SIGNIFICANCE

At the very start of our consideration of plant nutrition we must deal with the wonderful process which represents the most important and, at the same time, the most difficult problem in the realm of biochemistry. It is also the most characteristic feature of plants. The nutrition of green plants by means of light consists in the absorption and assimilation of atmospheric carbon dioxide by the green parts of plants whereby the carbon remains in the plant

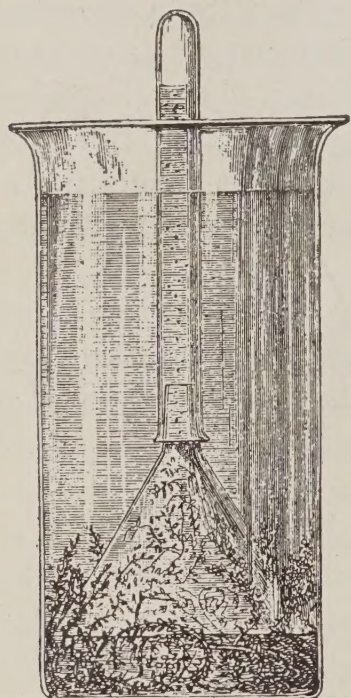


FIG. 6.—Ingen-Housz's experiment. An illuminated water plant liberates bubbles of gas rich in oxygen. (After Palladin.)

and the oxygen is returned to the atmosphere. Thus the CO_2 is decomposed into C and O_2 . This process involves the use of considerable energy which in nature is furnished by the radiant energy of the sun. de Saussure¹ and later Boussingault² showed that simultaneously with the assimilation of carbon, the elements of water are also incorporated into the substance of plants. Moreover, plants absorb small amounts of ash constituents and nitrogenous compounds from the soil. From all these raw materials plants synthesize organic compounds and for these endothermal reactions make direct use of solar energy. The direct dependence of the release of oxygen by plants on the light was proved by the following classical experiment of Ingen-Housz.³

If a glass beaker, filled with water containing carbon dioxide or, better still, a bicarbonate, is set in a bright light, and if in the water is immersed a small bundle of twigs of a water plant in such a way that the cut ends point upward, and these are covered by a funnel with the stem leading into a test tube filled with water (Fig. 6), it will soon be noted that streams of gas bubbles are rising from the cut ends of the plants and collecting in the test tube. This gas is very rich in oxygen. If a glowing splint is introduced into a tube full of the gas, it bursts into flame. The other gases which enter the test tube with the oxygen are chiefly nitrogen and carbon dioxide which were forced from the

¹ de Saussure, Th. *Recherches chimiques sur la végétation*. 1804.

² Boussingault, J. B. *Agronomie, chimie agricole et physiologie*. 1860-1878.

³ Ingen-Housz, J. *Experiments upon vegetables*. 1779.

intercellular spaces along with the oxygen. When the vessel is placed in a weak light the stream of bubbles becomes slower. Boussingault¹ performed numerous experiments in which he used the luminescence of phosphorus as a test for oxygen. He showed that the liberation of oxygen starts in the light and stops instantly in darkness.

Boussingault² showed that the volume of the oxygen released is practically equal to that of the carbon dioxide absorbed. Later investigators³ found deviations in this equality but recently, by using exact methods of gas analysis it has been established⁴ that when green leaves are functioning normally the ratio of the oxygen to the carbon dioxide is exactly 1, as the law of Avogadro would indicate, since the gas exchange proceeds in an equimolecular ratio.

The supposition that hydrogen also escapes in the gas exchange⁵ seems to rest on a misunderstanding.

This gas exchange is a part of the grand cosmic process which links life on earth with its fountain head—the sun. In every organism there are uninterrupted processes which liberate or transform energy. It follows that every living thing, like a complex chemical factory with which it is so often compared, must constantly have at its disposal a store of potential energy at high tension because transformations of energy are possible only at a certain potential. For example, it is easy to change to kinetic energy the heat energy contained in a glass of hot water. If a glass dish of ether is immersed in this water, the ether boils and the resulting vapor pressure will run a small motor. But if the same glass of hot water is poured into a barrel of cold water it is no longer possible to change the heat energy to kinetic energy although there has been no loss of heat. The heat of the hot water has been evenly distributed in the cold water and the latter was not perceptibly warmed by it. By such a dispersion of heat energy, its potential has fallen so low that it can no longer be utilized.

In the vital processes of every organism there is a constant dispersion of energy which, from the physiological standpoint, amounts to a total loss of energy (entropy). To a large extent this is also true of inanimate nature so that our earth in its flight through space liberates the depreciated energy of its physico-chemical processes and thus takes part in the increase of entropy in the universe. So it is necessary to replace the energy lost and the sun is the only source of this replacement.

The high-potential solar energy is stored in green leaves as the author of the first principle of thermodynamics, J. R. Mayer, correctly stated. In this wonderful apparatus the energy of the sun's rays is caught and "desposited"

¹ Boussingault. *Ann. des. Sci. Nat.* (5) 10: 330. 1869.

² Boussingault. *Agronomie, chimie agricole et physiologie.* 3: 266 and 378. 1864; 5: 1. 1874.

³ Pfeffer, W. *Arb. d. bot. Inst. in Würzburg.* 1: 31. 1871; Godlewski, E. *Flora.* P. 349. 1873; cf. especially Bonnier et Mangin. *Compt. rend.* 100: 1303. 1885; Idem. *Ann. des Sci. Nat. de Bot.* (7) 3: 5. 1886.

⁴ Maquenne et Demoussy. *Nouvelles recherches sur les échanges gazeux des plantes vertes avec l'atmosphère.* P. 118. 1913; Willstätter und Stoll. *Untersuchungen über die Assimilation der Kohlensäure.* P. 315. 1918; Kostytschew. *Ber. d. Bot. Ges.* 39: 319. 1921.

⁵ Pollacci, G. *Atti dell' intit. bot. Pavia.* Vol. 8: 1902.

(*abgelagert*) in the form of the chemical energy of organic compounds. Solar energy can remain in this condition for centuries. An example is provided by the coal deposits which are the residues of dead plants of previous geological periods. This solar energy is set free again after hundreds of thousands of years when it moves our ships and factories.

Since the entire animal world is fed only with organic substances which in the final analysis were once parts of plants, the animals also live at the expense of the stored energy of the trapped rays of sunlight. The sunbeam by a single process activates the powerful muscles of a rushing race horse, the hand of a great artist and the screws of a giant ocean liner which plows through the ocean waves. All this takes place through the agency of plants.

2. HISTORICAL REVIEW

The great British chemist, Joseph Priestley, whom we must thank for the discovery of oxygen, hydrochloric acid, ammonia, carbon monoxide and sulphur dioxide, noticed that the atmosphere was "rectified" by certain cosmic processes after it had been "spoiled" by animal respiration or by the burning of something in it. At first he looked for an effect of seas and oceans on the atmosphere because it obviously required some process on a large scale to balance animal respiration and combustion in general. Simple experiments soon convinced him that air spoiled by respiration remained unfit for respiration after repeated shaking with water. Then Priestley turned to experiments with plants which gave positive results. Thus Priestley once placed a mouse under a bell-jar which was inverted with its rim immersed in water. The mouse by its respiration lowered the vitality of the air to the point where it could no longer live in it and a lighted candle was extinguished in this air. Then Priestley placed a little branch of mint under the jar and left it there for several weeks. The result was that the air was rendered fit for both respiration and combustion.

Priestley's experiments created a great sensation. For them he was given, among other things, the gold medal of the Royal Society of London. Even then the full meaning of the discovery was unknown to this skilled investigator. Begun as the problem of the chemical composition of the atmosphere, it helped to solve the more important question of the energy connection between the sun and life on earth. But Priestley did not study the conditions under which plants rectify the air. This is why he was helpless against the objections of another celebrated chemist—Scheele—who repeated Priestley's experiments and obtained contradictory results. Scheele found that plants spoiled the air just as animals do. Priestley later obtained the same results and could not explain the discrepancy. He decided to give up his whole theory of the antithesis between the plant and animal world.

The problem was first solved by the famous Dutch scientist, Jan Ingen-Housz.¹ In a series of experiments, which for his time were extraordinarily exact, profound and critically thought out, Ingen-Housz laid the foundation of the knowledge of the nutrition of plants through light waves which is now one of the best established facts in the realm of plant physiology. Priestley claimed that the air was improved by the growth of plants in it. Ingen-Housz proved that this was not correct; only the green parts of plants improve the air and then only in the light, for in darkness plants respire like animals.² The action of green plants consists in the utilization of solar energy to take from the air a nutrient substance containing oxygen and to return the oxygen to the atmosphere in molecular form. When Lavoisier did away with the phlogiston theory and set up the doctrine of the oxides, Ingen-Housz leaned on the new theory in order to make his conclusions perfectly clear. The plants assimilate the carbon dioxide from the air and release oxygen to the air. In darkness the reverse process takes place but to a lesser extent. The organic compounds are oxidized during respiration and

¹ Ingen-Housz. *loc. cit.*

² This explained the discrepancy in the results of Priestley and Scheele. These two workers performed their experiments under unlike conditions of illumination.

their carbon passes into the air in the form of carbon dioxide. In this way Ingen-Housz laid the foundation for the theories of nutrition through light and of the respiration of plants. Hence he is to be regarded as the founder of chemical plant physiology.¹ At this time experimental technique was on a low plane and the present status of this question is based on the classical work of Th. de Saussure.² He furnished the quantitative proof for the chemical equation of photosynthesis and also showed that plants in the light assimilate the elements of water as well as carbon. He also laid the foundations for the present conception of plant respiration and brought out many other fundamental facts, so that his service was no less valuable than that of Ingen-Housz.

Finally we are indebted to the great French agricultural chemist—J. B. Boussingault³—for his very exact studies of the nutrition of plants, especially in the field of the assimilation of nitrogen. Boussingault was the first to introduce the practice of growing plants in cultures on pure mineral nutrients.

3. PHOTOCHEMICAL REACTIONS IN GENERAL

We must now study more carefully the great photochemical reaction which is found in green leaves and, for better orientation, state the most important laws which apply to photochemical processes in general.

Unlike heat energy, the energy of light waves does not affect all chemical reactions but only those with substances that are sensitive to light and which change the energy of light not only into heat energy (as do substances insensitive to light) but also into chemical energy. A characteristic property of these substances is their fluorescence. It has recently been established that many reactions, which do not ordinarily depend on light, become photochemical in the presence of suitable light receptors.

The first fundamental law of photochemical reactions states that *only absorbed rays exert a chemical effect* (Draper). Many substances which are sensitive to light are not sufficiently active because they absorb very little light. In these cases the chemical potential is considerably increased by the presence of sensitizers, *i.e.*, pigments which absorb much light of certain wave lengths and pass it on to other substances.

The foregoing law is a direct consequence of the first principle of thermodynamics. Its quantitative statement says that *the photochemical effect is directly proportional to the light energy absorbed*. This is the second law to be kept in mind in judging the processes in green leaves.

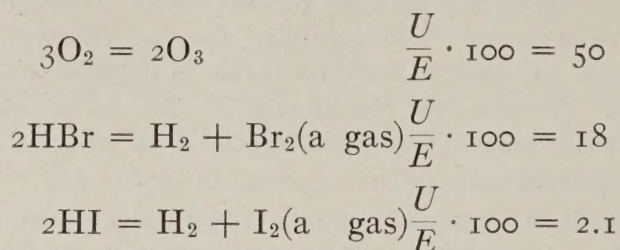
The third law states that *a certain percentage of the light energy absorbed is used for chemical action* (Bunsen). If we take E for the radiant energy absorbed and U for the chemical work done, the ratio $\frac{U}{E} \cdot 100$ is the measure of

¹ Through his brilliant discoveries Ingen-Housz straightened out many difficulties. In the first place, Priestley's pride was sorely hurt because he overlooked a great discovery and another explained his error. In his prejudiced way, Priestley carried on a long controversy with Ingen-Housz whose results he partly questioned and partly appropriated for himself. The Italian scholar Cavallo and the Geneva priest Senebier sided with Priestley. Senebier also described Ingen-Housz's discoveries as his own. Consequently the name of Ingen-Housz was for a century either placed in the background or mentioned incidentally along with those of Priestley, Senebier and others. The true facts have only recently come to light. Cf. Treub. J. Ingen-Housz (1880) and especially J. Wiesner. J. Ingen-Housz (1905).

² de Saussure, Th. *loc. cit.*

³ Boussingault. *Agronomie, chimie agricole et physiologie*. 1860-1878.

efficiency of a process. For the wave length $209\mu\mu$, E. Warburg¹ found the following values of $\frac{U}{E} \cdot 100$ for different photochemical reactions:



The chemical effect is directly proportional to the intensity of the light and to the time. Photochemical reactions are largely oxidations and especially reductions. It may be added that the most important photochemical reactions in green plants are reductions.

The question of the specific nature of the effect of different regions of the spectrum is not wholly clear. There is no doubt that photochemical reactions are possible under the influence of any rays but whether the photochemical yield is equal remains to be shown. According to the quantum theory (see below) this must become smaller with decrease in wave length. Results consistent with this theory have been obtained in the case of photochemical cleavage of hydrogen bromide and iodide² and in photosynthesis (see p. 103).

As for the intensity of the light in the different regions of the spectrum, this is a result of physiological sensitivity and is therefore not an objective measure of the radiant energy. Many truly active rays are not even visible to our eyes.

An interesting feature of photochemical reactions is their specific temperature coefficient. The van't Hoff rule (described in the first chapter) states that the reaction velocity is doubled, tripled or even more affected for each temperature interval of 10° (within certain limits). Photochemical reactions are exceptions to this rule in that they have a temperature coefficient no higher than 1.1-1.2. A higher temperature coefficient of a photochemical process means that it is a complex process in which reactions other than photochemical are involved.

It is known that every chemical substance has a definite absorption spectrum. On the basis of the present condition of atomic physics *we consider the absorption spectrum to be a function of the molecular and atomic structure of the substance concerned.* The absorption spectrum may come in the invisible as well as in the visible part of the solar spectrum. The sun sends us rays of the wave lengths $300-6000\mu\mu$ but our eyes see as light only the wave lengths $390-760\mu\mu$.

From the standpoint of the quantum theory, the photochemical effect must be greater in the less refrangible rays than in the more refrangible ones, since according to Einstein's law of equivalence, in the case of photochemical transformations each substance absorbs the radiant energy in quanta and at the rate of one quantum per molecule. Each quantum equals $h\nu$, where h is Plank's

¹ Warburg, E. Zeitschr. f. Electrochem. 26: 54. 1920.

² Warburg, E. *loc. cit.*

constant and ν is the rate of vibration of the light wave concerned. For example, per gram calorie there is a considerably greater number of quanta in the red rays than in the violet rays. This means that the number of quanta and not the value of single quanta determines the photochemical yield. Hence the exchanges of energy φ in different regions of the spectrum are proportional to the wave lengths λ :

$$\frac{\varphi_1}{\varphi_2} = \frac{\lambda_1}{\lambda_2}$$

This regularity is illustrated by the following table of E. Warburg:

λ	207 $\mu\mu$	253 $\mu\mu$	282 $\mu\mu$	486 $\mu\mu$	589 $\mu\mu$	800 $\mu\mu$
φ	0.7286	0.8904	0.9924	1.710	2.073	2.816

From this table it is evident that when $\lambda = 800\mu\mu$ the photochemical yield for the same consumption of energy is about four times as great as when $\lambda = 207\mu\mu$. On the contrary, the total energy absorbed by 1 mol of substance is correspondingly greater at $\lambda = 207\mu\mu$ than when $\lambda = 800\mu\mu$. At $700\mu\mu$ a quantum ($h\nu$) equals 5.99×10^{-20} gram calories.

4. THE GENERAL NATURE OF THE LEAF PIGMENTS

From the foregoing statement it is evident that the attention of scientific investigators has been rightly given to the pigments contained in leaves, since the photochemical process of photosynthesis (synthesis of organic substances) goes on at the expense of light absorption by means of these pigments. Experiments to isolate and purify the leaf pigments and to learn their chemical nature have been attempted for years but it is only recently that significant results have been obtained.

In the 17th century Grew¹ found that the green pigment could be extracted from plants with alcohol. Later this pigment came to be known as *chlorophyll*.² The alcoholic extract of leaves has a Guignet's green color with a strong blue-red fluorescence. It contains not merely one green pigment for the celebrated British physicist Stokes³ found that there are always two green and two yellow pigments in leaves. Only recently have the pioneer studies of Willstätter, of which more will be said later, proved beyond doubt the entire accuracy of the neglected results of Stokes' work.

If benzene or petrol ether is added to the alcoholic extract and the mixture shaken and allowed to stand for some time, the green pigments pass into the upper layer of benzene and the alcohol then has only a yellow color. This characteristic reaction is called the Kraus reaction.⁴

¹ Grew, N. *Anatomy of Plants*. P. 273. 1682.

² Pelletier et Caventou. *Ann. de chim. et de physique* (2) 9: 194. 1818.

³ Stokes, G. G. *Proc. Roy. Soc. London*. 13: 144. 1864; *Idem*. *Jour. Chem. Soc. (London)* 17: 304. 1864.

⁴ Kraus, G. *Zur Kenntnis d. Chlorophyllfarbst.* 1872; see also Sorby, H. C. *Proc. Roy. Soc. London*. 15: 433. 1867; 21: 442. 1873. The name "Kraus reaction" is incorrect because Stokes discovered the principle of the method.

The yellow pigment which colors the lower layer is called *xanthophyll*. For many plants this method gives opposite results or the so-called reversed Kraus reaction;¹ the upper layer becomes yellow and the lower one remains green. Between the normal and reversed Kraus reaction various transition stages have been found. There is a possibility of a reversed reaction because there are in leaves at least two yellow pigments which are unequally soluble in benzene and alcohol. The one more easily dissolved in benzene is of an orange-yellow color. Carrot roots and many fruits show this color. Since it was first taken from carrots it bears the name *carotin*. The irregular results of the Kraus reaction led to the conclusion² that the green pigments may be divided between the benzene and alcohol but later it turned out that the green pigment which remains in the alcohol is a decomposition product of the naturally labile substance. The two genuine green pigments pass to the upper layer of benzene.

A quantitative separation of the green and yellow pigments of the leaf extract can be made by chromatographic methods.³ These methods depend on the differential adsorption of the several components of the extract. It is best to extract the leaves with carbon disulphide and to filter the solution through a glass tube filled with finely powdered chalk or some other adsorbent. The several pigments are adsorbed at different layers in the tube and there show the corresponding colors. Carotin is not adsorbed and passes through. The different colored zones of the column of calcium carbonate are then removed one by one with a scalpel and from each section a single pigment is extracted with the usual solvent.

According to Tswett, there are in leaves in addition to chlorophyll A and B and carotin, not one but four xanthophylls which differ in their adsorption and in their absorption spectra. Chemical investigation has not confirmed this view. Perhaps all of Tswett's xanthophylls are isomeric and isomorphic or, more likely, they represent artifacts or different physical conditions of a single substance. Both the chromatographic method and the appearance of the absorption bands in the spectrum are insufficient to prove the chemical difference of the substance.⁴

The yellow pigments in autumn leaves were once identified with the normal yellow pigments of green leaves. Now it has been proved that, in addition to carotin and xanthophyll in the yellow leaves of autumn, there are many other pigments which do not appear in green leaves.⁵ Many of them are soluble in water. The quantities of carotin and xanthophyll in yellow autumn leaves are often quite small. A somewhat larger amount of carotin appears in the red leaves.⁶

¹ Monteverde, N. *Acta Horti Petropolitani*. 13: 123. 1893.

² Monteverde, N. *loc. cit.*

³ Tswett, M. *Ber. d. bot. Ges.* 24: 316 and 384. 1906; *Idem*. *Chromophyll in the plant and animal worlds*. 1910 (Russian).

⁴ Willstätter und Stoll. *Untersuchungen über Chlorophyll*. P. 235. 1913, [translated by Schertz in 1928].

⁵ Lubimenko, W. *The transformations of the plastid pigments in living plant tissues*. 1916 (Russian); Görrig, E. *Beihefte z. bot. Centralbl.* (I) 35: 342. 1918; Willstätter und Stoll. *Untersuchungen über Assimilation der Kohlensäure*. P. 27. 1918.

⁶ Molisch, H. *Ber. d. bot. Ges.* 20: 442. 1902.

In the young leaves of plants germinated in darkness and still yellowish in color, the yellow pigments of green leaves are already present¹ but in addition there are other water-soluble pigments. Since these "etiolated" (without chlorophyll) plants are not assimilating CO₂ in the light the assumption is not precluded that the yellow pigments have nothing to do with photosynthesis.² Their physiological importance has not been made clear.

It would be very desirable to learn the origin of carotin and xanthophyll in leaves. At present the question is unsettled and we have in the main various teleological considerations³ which are of no assistance.

5. CHLOROPHYLL

The one undoubtedly light-sensitive substance which takes part in photosynthesis is chlorophyll. It is the most interesting organic substance in nature because only through its direct or indirect action are all other organic substances synthesized. It is easy to understand why many students are occupied in learning the physical and chemical properties of this substance. In no other field of biological chemistry do we find such wasted efforts and contradictory results. The history of the problem of chlorophyll is instructive from the standpoint of methods but it cannot be given here. That the careful work of many capable students has borne so little fruit is due to the uncommon instability and other properties of chlorophyll which is a very difficult substance to study. Since these particular difficulties appear in many other biochemical studies, they deserve a brief explanation. The purification of chlorophyll, proteins and other colloidal substances is rendered difficult not only by their instability but also by the fact that the usual methods of organic chemistry cannot be used to purify them. Moreover, it is not always possible to calculate the correct formulae for pure substances of high molecular weights, by analysis of the elements, because the normal errors of analysis (in per cent) amount to one or even several atoms of the various elements. Likewise it is usually impossible to determine the molecular weight with sufficient accuracy by the usual methods if the molecule is very large and the purity of the preparation is uncertain. In view of these serious obstacles the study of complex but biologically important substances of high molecular weight is usually begun by an attempt to split the large molecule. The cleavage products are frequently crystalline substances which can be purified and studied by the usual methods. The study of the chemical nature of these cleavage products and the proof of their constitution gives a starting point in estimating the nature of the original large molecule. Subsequent synthesis of it from the cleavage products completes this part of the work.

Although this method has been known for a long time, Willstätter first applied it to the study of chlorophyll and at once obtained such new, reliable results that all of the work of his predecessors is now of historical but no actual

¹ Kohl. Untersuchungen über das Carotin. 1902; Lubimenko. *loc. cit.*

² On this point see especially Willstätter und Stoll. *loc. cit.*

³ Stahl. Zur Biologie des Chlorophylls. 1909; Iwanowsky, D. Mitt. d. Warschauer Univ. Vol. 1. 1914 (Russian).

interest. However, it would be wrong to suppose that Willstätter has used a previous method without modifications. He has introduced a new and very essential improvement. The chlorophyll has been split according to the rule. By the use of ingeniously selected reactions he has removed from the chlorophyll molecule one cleavage product after another. The molecular weight of the residue gradually becomes smaller and its stability greater. It is especially important that loss of cleavage products has been prevented, since each cleavage has been checked by synthesis of the original substance from the cleavage product and the residue. If the molecule had been broken into several parts the control would have indicated it. In order to be still more certain of his results Willstätter took the chlorophyll apart in two different ways so that the substances in one series could be transformed into those of the other series.

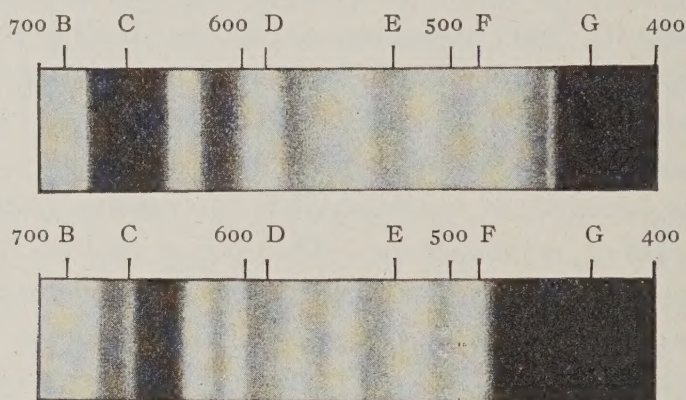


FIG. 7.—The spectrum of chlorophyll *a* (above) and that of chlorophyll *b* (below). (After Willstätter and Stoll.)

It is evident that Willstätter's work with chlorophyll has pointed out some very valuable methods. His results will serve as a standard for biochemical studies of other compounds of high molecular weight.

The physical properties of chlorophyll are more easily studied and are very interesting. Hence they will be described first.

The Physical Properties of Chlorophyll.—It has already been mentioned that leaves contain two green pigments which closely resemble each other in their properties and composition. Both form microscopic crystals,¹ insoluble in water but easily dissolved in alcohol, acetone, chloroform and carbon disulphide.

In alcoholic solution, chlorophyll *a* is a blue-green substance with a ruby-red fluorescence. Chlorophyll *b* is bright green in alcohol and its fluorescence is brownish-red. Chlorophyll solutions in organic solvents soon become brown in the light and are finally entirely bleached; but if enough water is added to the solution to make an emulsion of chlorophyll, the bleaching is considerably retarded, especially in the presence of colloids.²

¹ Willstätter und Stoll. *Liebigs Ann. d. Chem.* 390: 327. 1912.

² Iwanowsky, D. The physical condition of chlorophyll in living leaves. 1913 (Russian); Willstätter und Stoll. *Untersuchungen über Assimilation der Kohlensäure*. P. 309. 1918; Wurmser, R. *Compt. rend. soc. de biol.* 83: 437. 1920.

The absorption spectrum¹ of the green pigments is particularly interesting in connection with their physiological rôles of sensitizers. The spectra are shown in Fig. 7. Their chief characteristic is the strong absorption band in the red part of the spectrum between the [Fraunhofer] lines *B* and *C*. The green color of chlorophyll is largely due to the absorption of the red rays. The spectrum analysis shows that only certain red rays are absorbed. Those at the left of the line *B* are transmitted even at high concentrations of the pigments. The absorption band between *B* and *C* is very characteristic of chlorophyll and is the main determiner of its physiological function. Chlorophyll has even been searched for on other planets on the basis of the occurrence of this band.² For chlorophyll *b* this band consists of two separate strips. In very dilute solutions of chlorophyll there appear still other bands as follows:

- II. between *C* and *D* in the orange (for chlorophyll *b* this band is also divided into two parts),
- III. to the right of the line *D* in the yellow,
- IV. on the line *E* in the green,
- V. beside the line *F* in the blue,
- VI. between the lines *F* and *G* (weak absorption by chlorophyll *a*, strong absorption by chlorophyll *b*),
- VII. a continuous absorption to the right of *G*.

These bands are separate only in weak concentrations of the pigments. At strong concentrations, which depend on the condition of the chlorophyll in the leaf, the principal band between *B* and *C* widens rapidly toward the right. At a concentration of the solution corresponding to the content of chlorophyll in the plastids there is a widening of the band near *E*; the two bands in the blue-violet part of the spectrum and the one at the end fuse but widen only slightly toward the left (Fig. 8). In this way we have only two absorption bands in physiologically active chlorophyll, a broad one in the left (less refrangible) and a narrower one in the right (more refrangible) portion of the spectrum.

The spectrum of living leaves has often been studied (after injection of the intercellular spaces with water). In this spectrum all the absorption bands are moved to the left and the order of their strength is not the same as in solution. Careful investigations have shown that chlorophyll exists in living leaves either in the solid form³ or, perhaps more correctly, in the form of a colloidal solution.⁴ It has been shown recently⁵ that chlorophyll is found in leaves in a molecular, lipoidal solution.

Willstätter and his colleagues used the spectrum analysis of chlorophyll to test the purity of their preparations. It showed whether there had been any decomposition or alteration of the pigment. They were very careful to

¹ Tswett, M. *loc. cit.*; Willstätter und Stoll. Untersuchungen über Chlorophyll. 1913, [translated by Schertz in 1928].

² Arzichowsky, W. Ber. d. Donschen polytech. Inst. Vol. I. 1912 (Russian).

³ Iwanowsky, D. Ber. d. bot. Ges. 25: 416. 1908; Idem. Biochem. Zeitschr. 48: 328. 1913.

⁴ Herlitzka. Biochem. Zeitschr. 38: 321. 1912; Willstätter und Stoll. Untersuchungen über Chlorophyll. P. 60. 1913, [translated by Schertz in 1928].

⁵ Stern, K. Ber. d. bot. Ges. 38: 28. 1920; Idem. Zeitschr. f. Bot. 13: 193. 1921. According to K. Noack (Biochem. Zeitschr. 183: 135. 1927), chlorophyll is adsorbed in molecular solution.

isolate an unchanged pigment and they failed in their first attempts. As a rule, the constancy of the optical properties can hardly be a reliable criterion. The absorption spectrum may not be a reliable physical constant. As we have already seen, considerable variations of the spectrum of a single chemical substance may depend on its physical condition and, on the contrary, important chemical modifications might not affect the spectrum if the chromophoric groups were uninjured. Hence, it is dangerous to identify pigments by their spectra.

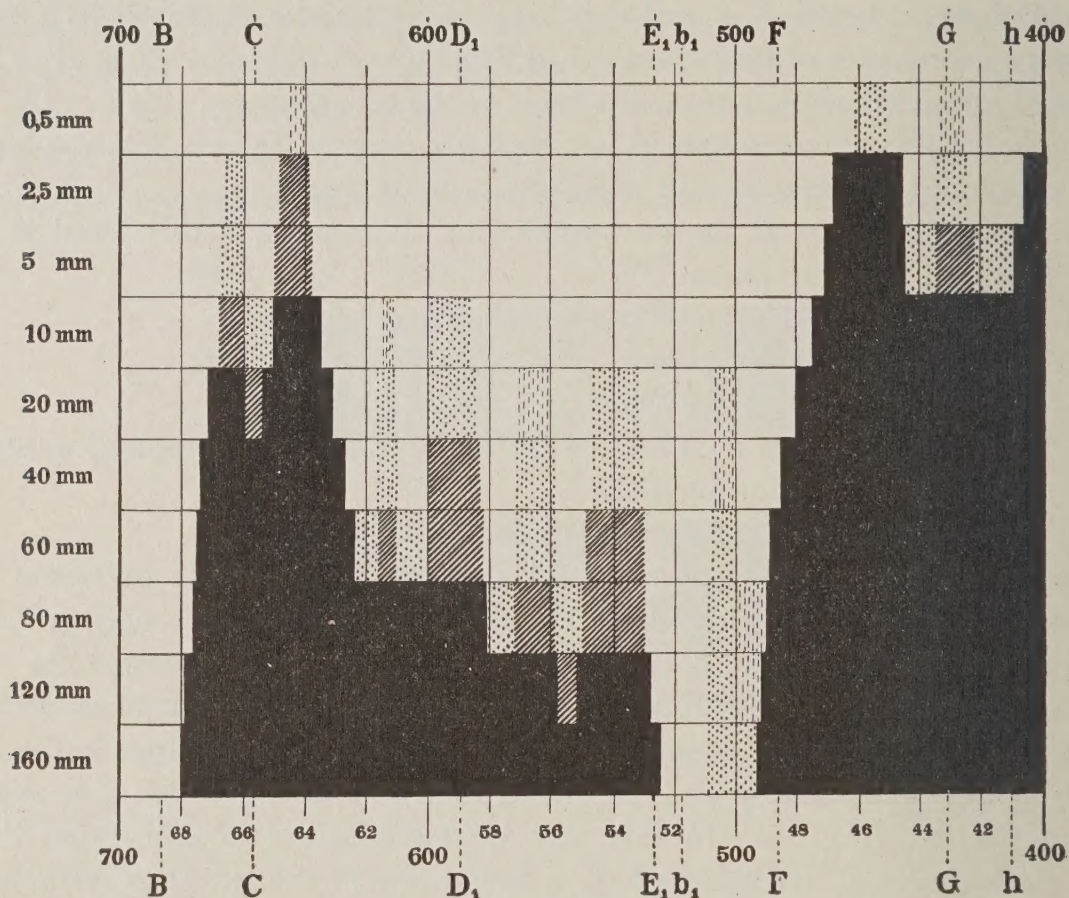


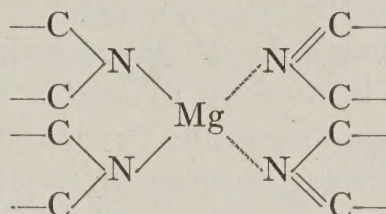
FIG. 8.—The fusion of the absorption bands of chlorophyllin with increase in concentration of its solution. At very high concentrations only the extreme red and the green rays are left unabsorbed. (After Willstätter and Stoll.)

The Chemical Properties of Chlorophyll. The investigations of Willstätter and his colleagues¹ were carried out by the method of splitting the complex chlorophyll molecule by stages. In one series of experiments the cleavage was brought about by acids and in the other series by alkalies. It was found at once that the two series of products differed only by the presence or absence of magnesium. The use of alkali gave substances containing magnesium and its percentage content increased as the molecule was decomposed, since the magnesium is not removed by boiling with strong alkali or even by fusion with soda lime. The action of the acids removed the magnesium immediately. This takes place even when a weak organic acid is applied without raising the temperature. If the magnesium is removed from the products of the alkali action, these substances become identical with the corresponding products of the action

¹ Willstätter und Stoll. Untersuchungen über Chlorophyll. 1913, [translated by Schertz in 1928]. The earlier work of Willstätter and his colleagues is cited here.

of acids and vice versa. There are no other ash substances in chlorophyll. Earlier workers presented proofs of the presence of iron and phosphorus in chlorophyll but this is now disproved.

Magnesium is associated only with atoms of nitrogen in the chlorophyll molecule, as it has been shown to do in analogous substances.¹ It is never united to oxygen. Magnesium is linked by both its valences to the two nitrogen atoms of pyrrol and is united in a complex way with the two other nitrogen atoms:²



Willstätter is inclined to attribute great physiological significance to the presence of magnesium in chlorophyll because he ascribes to it, by analogy with the substances discovered by Grignard,³ the rôle of a synthesizing factor in the process of assimilation and not merely that of a sensitizer. According to Grignard's principle, syntheses have actually been produced by substances in which magnesium is associated with nitrogen,⁴ but this is only an analogy.

It is interesting that the removal of the magnesium brings a sharp change in the optical properties of the pigment. The Guignet green becomes a brownish- or olive-green and the fluorescence almost entirely disappears. Since the magnesium is not removed by treatment with alkali, the green color and the fluorescence remain even when the chlorophyll molecule has been highly modified.

Magnesium is removed from chlorophyll by the action of weak oxalic acid and even carbonic acid⁵ and is replaced by two atoms of hydrogen. The resultant substance has an olive-brown color, a waxy consistency and the properties of an ester. It is called phaeophytin and it is very interesting that it easily forms complex compounds with metals such as iron, zinc and copper. These compounds are also green and have a strong fluorescence, like chlorophyll. This is a very sensitive test for zinc and copper. Magnesium has been replaced in phaeophytin with the greatest difficulty. Willstätter did it by Grignard's method and thus proved that phaeophytin actually differs from chlorophyll only by the absence of magnesium.

There are two alcohols held in the ester union in the phaeophytin molecule. When completely saponified, both alcohols are removed. One of them is ordi-

¹ Tschugajew, L. Ber. chem. Ges. 40: 1973. 1907.

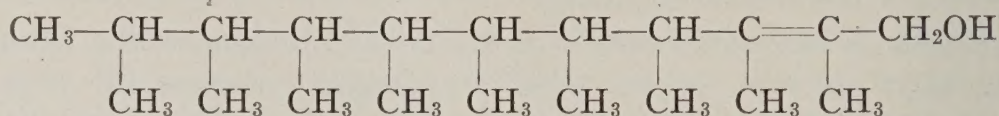
² Werner, A. Neuere Anschauungen auf dem Gebiete der anorganischen Chemie. 1909.

³ Grignard. Compt. rend. 130: 1322. 1900; Grignard et Tissier. Ibid. 132: 835. 1901; Grignard. Ann. de chim. et de physique (7) 24: 483. 1901; and many others.

⁴ Oddo, B. Ber. chem. Ges. 43: 1012. 1910. The carbon atom adjacent to the nitrogen is thereby made a part of a chain.

⁵ Willstätter und Stoll. Ber. chem. Ges. 50: 1791. 1917; Idem. Untersuchungen über die Assimilation der Kohlensäure. P. 226. 1918; Jorgensen and Kidd. Proc. Roy. Soc. London (B) 89: 342. 1917.

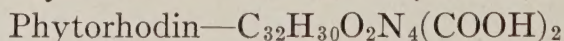
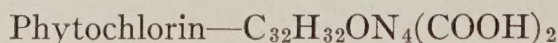
nary methyl alcohol and the other is a branched carbon chain of the empirical composition $C_{20}H_{39}OH$ and the following constitution:



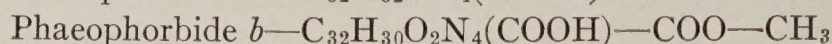
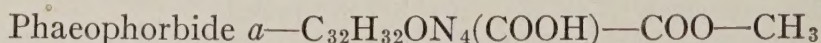
This substance is called phytol. It is a viscous colorless liquid. The numerous methyl groups gives it a high capacity for oxidation, and it is changed when allowed to stand exposed to the air. The great merit of the new method of investigation is evident in that, although phytol makes up a third of the chlorophyll molecule, it was not isolated by the earlier investigators. The strong oxygen absorption of phytol may possibly be connected with the reducing function of chlorophyll. The residue of phaeophytin after the removal of phytol is called phaeophorbide and has acid properties. Its solutions in organic solvents are olive-brown in color.

The substances which are obtained from chlorophyll *a* and *b* differ so little from each other that here they are not described separately but are designated as phaeophytin *a* and *b* or phaeophorbide *a* and *b*. However, it is evident that if these complexes are removed from two unlike molecules, there will be a difference between the parts that are left. The actual result of complete saponification of phaeophytin (*i.e.*, when both methyl alcohol and phytol are removed) is dicarboxylic acids which do differ clearly from each other. From chlorophyll *a* we get phytochlorin of an olive-green color in ether solution and from chlorophyll *b* we obtain phytorhodin which is red with a faint tinge of violet (also in ether solution). Their acid properties are characteristic of both substances and both form interesting complex compounds with potassium. The fact that phaeophytin is formed from two different products was a proof of the double nature of chlorophyll itself. Since the relative amounts of chlorophylls *a* and *b* are not always the same for different plants, it is easy to imagine what a puzzle it was for Willstätter to analyze the phaeophytins and phaeophorbides without knowing that he was dealing with a mixture of two different substances.

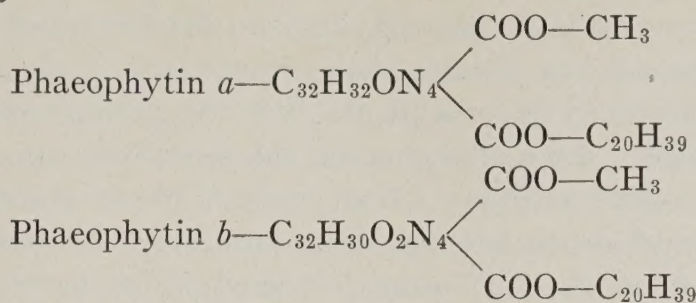
The isolation of two fairly stable acids—phytochlorin and phytorhodin—from different plants made it possible to determine the composition of these substances. Analysis gave the empirical formulae:



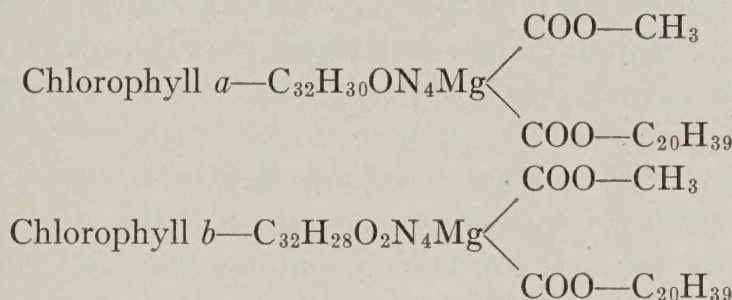
Since the first came from chlorophyll *a* and the second from chlorophyll *b*, after these formulae were determined it was possible to arrive at the composition of the primary substances. They proved to have the formulae:



and consequently:



If into the formula of these phaeophytins we introduce an atom of magnesium in place of two atoms of hydrogen we obtain the formulae for the true chlorophylls:



The further cleavage of phytochlorin and phytorhodin is carried out in the same way as the cleavage of the products of the action of alkali on chlorophyll.

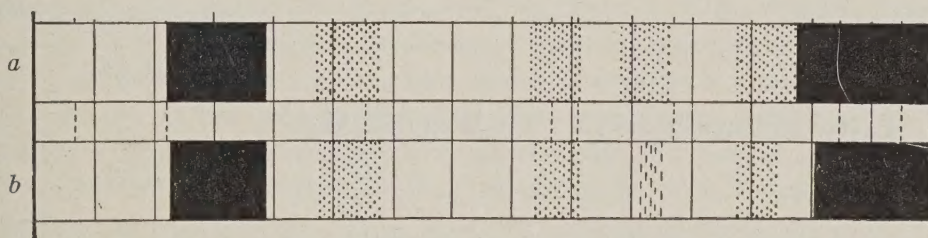
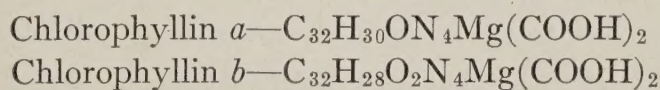


FIG. 9.—(a) Absorption spectrum of complex potassium compounds of phytochlorin. (b) Absorption spectrum of chlorophyllin. (After Willstätter and Stoll.)

This cleavage will now be described. The esters are saponified by the action of lye and there are formed salts of dicarboxylic acids—chlorophyllins *a* and *b*, which differ from phytochlorin and phytorhodin (*cf.* their formulae) only by the presence of an atom of magnesium in the molecule:

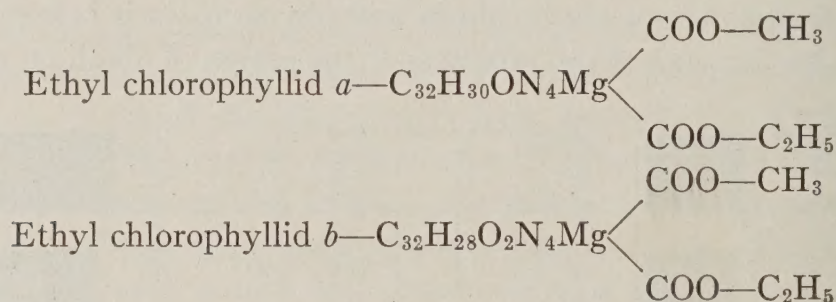


The chlorophyllins have the color of natural chlorophyll from which they also differ little in absorption spectrum. It is very remarkable that the absorption spectra of the chlorophyllins are identical with those of the complex potassium compounds of phytochlorin and phytorhodin (Fig. 9). This shows that substances of different chemical composition may have the same absorption spectrum.^m

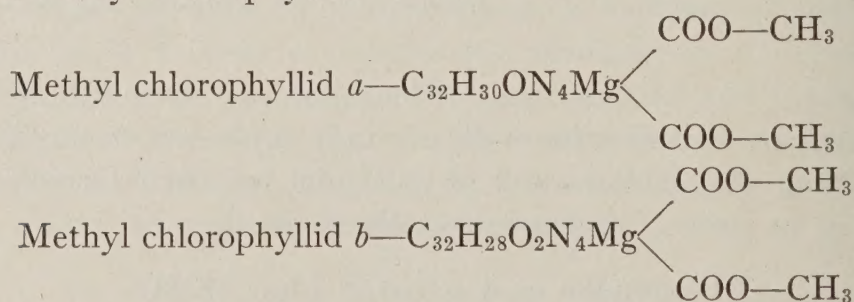
^m Other data and absorption bands for chlorophyll and its derivatives are given by Triebs and Wiedemann. Liebigs Ann. d. Chem. 466: 264-291. 1928.—Ed.

In order to obtain an incomplete saponification of chlorophyll and split off only phytol, use is made of chlorophyllase, an enzyme which is very common in leaves. The quantity of enzyme varies from plant to plant, wherefore the phytol is rapidly removed in some plants after the protoplasm is killed but in others it takes longer. Like all esterases, chlorophyllase also has a synthetic action under the proper conditions. If an excess of phytol is added to an acetone solution of chlorophyllid (the name given to the rest of chlorophyll after phytol is removed) and to it is added some powdered, dried leaves rich in chlorophyllase, there will be a synthesis of chlorophyll. Chlorophyllase loses its effect in the presence of large amounts of acetone, ether, alcohol and other organic solvents which are generally used to extract chlorophyll. However, its synthetic effect does appear in alcoholic extracts and the alcohol present in excess replaces phytol in the chlorophyll molecule. Thus phytol can be replaced by various alcohols with the resulting production of chlorophyll derivatives which do not occur naturally.

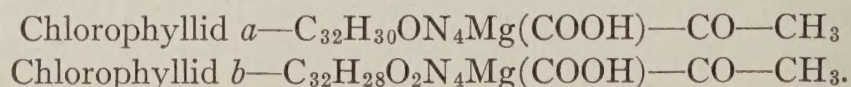
The so-called crystalline chlorophyll was discovered long ago¹ and found to appear as dark green crystals after evaporation of the alcoholic leaf extract. Willstätter showed that this substance is nothing but ethyl chlorophyllid, *i.e.*, chlorophyll in which the phytol is replaced by ethyl alcohol:



Willstätter has prepared the product of replacing phytol by methyl alcohol, the so-called methyl chlorophyllid:



Chlorophyllids with a free carboxyl group (analogous to the phaeophorbides) are obtained from chlorophyll by the action of chlorophyllase in ether solution or, still better, in acetone solutions:



Ethyl chlorophyllids and methyl chlorophyllids are dark, crystalline powders with a metallic sheen and the properties of esters. Their solutions in alcohol,

¹ Borodin, J. Bot. Zeit. 40: 608. 1882; Monteverde, N. *loc. cit.*

pyridine, ether, acetone and similar liquids are Guignet green in color. The free chlorophyllids *a* and *b* have acid properties. Under the influence of dilute acids they change easily into the phaeophorbides *a* and *b*, since the magnesium is then replaced by hydrogen. If either chlorophyll or any one of the chlorophyllids are saponified by alkali in the cold, the results are water-soluble salts of the chlorophyllins *a* and *b* (see above). Chlorophyllins are dicarboxylic acids, Guignet green in aqueous solution and in neutral organic solvents. The action of weak acids is to remove the magnesium and chlorophyllin *a* becomes phytochlorin while chlorophyllin *b* becomes phytorhodin. The reverse reactions take place when the smaller compounds are heated with methyl alcohol solutions of MgO and lye.

Only an exact study and careful analysis of each of these products made it possible for Willstätter and his colleagues to prove the identities of chlorophylls *a* and *b* in all the seed-plants and to establish the empirical formulae not only of the cleavage products but also of chlorophylls *a* and *b*, which were later isolated in pure form. The analysis of chlorophyll preparations from various plants disproved the assumption that there are different chlorophylls throughout the plant kingdom.¹

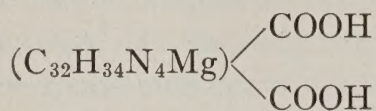
It has been difficult to determine the empirical formulae because both chlorophyll and its cleavage products are so unstable. Under the action of alkali there is a momentary change in color. Willstätter calls this allomerization and explains it as follows. One atom of nitrogen occurs in the molecule in the lactam group —NH—CO— . It is theoretically possible to have more of such groups in the molecule of chlorophyll and the naturally occurring product is the least stable; it saponifies easily and another atom of nitrogen unites with CO to form the stable lactam group. Chlorophyll itself may even be different mixtures of isomeric substances. In chlorophyllin, phytochlorin and phytorhodin the number of possible isomers is still greater because in these compounds all combinations between the nitrogen atoms and the three CO groups are conceivable. In fact one first obtains different kinds of phytochlorins and phytorhodins. By taking special precautions to prevent allomerization, it is possible to obtain uniform products which correspond to true chlorophyll and are called phytochlorin *e* and phytorhodin *g*. The instability of the natural lactam groups forms the basis of an excellent qualitative test for unchanged chlorophyll. This is the so-called "brown phase" test. Under the influence of alkali the green color at once turns to brown and in a few minutes returns to green, the changes depending on the alterations of the lactam groups. It is evident that these new stable lactam groups cannot give the brown phase again, so that the test with changed chlorophyll is negative.

The further cleavage of phytochlorin and phytorhodin, as well as of the chlorophyllins, is aimed at the removal of oxygen. For chlorophyll *a* this is done through the action of concentrated alkalis at increased pressure and high temperatures. All the oxygen is split off. For chlorophyll *b* an atom of carbon must be reduced in addition.

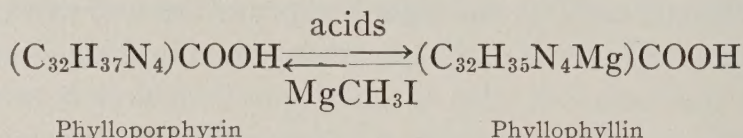
Oxygen is most easily split from the lactam groups and then from one of the free carboxyls. This is done by concentrated alkali in an autoclave at 140–190°. According to the temperature of the reaction, various products are thereby

¹ Etard, A. La biochimie et les chlorophylles. 1906; Gautier, A. Compt. rend. 120: 355. 1895; Idem. Bull. Soc. Chim. de France (4) 5: 319. 1909.

obtained from allomerized chlorophyllins. The first are those with two carboxyl groups, *e.g.*:



These are colored substances with characteristic spectra, and are generally known as phyllins. In the presence of strong alkalis, increased pressure and 190° , phyllin *a* splits off CO_2 and becomes two monocarboxylic acids, pyrophyllin and phyllophyllin, isomeric according to the position of the carboxyl groups. By treatment with acids, all the phyllins change to the corresponding porphyrins by the substitution of hydrogen for magnesium as usual:

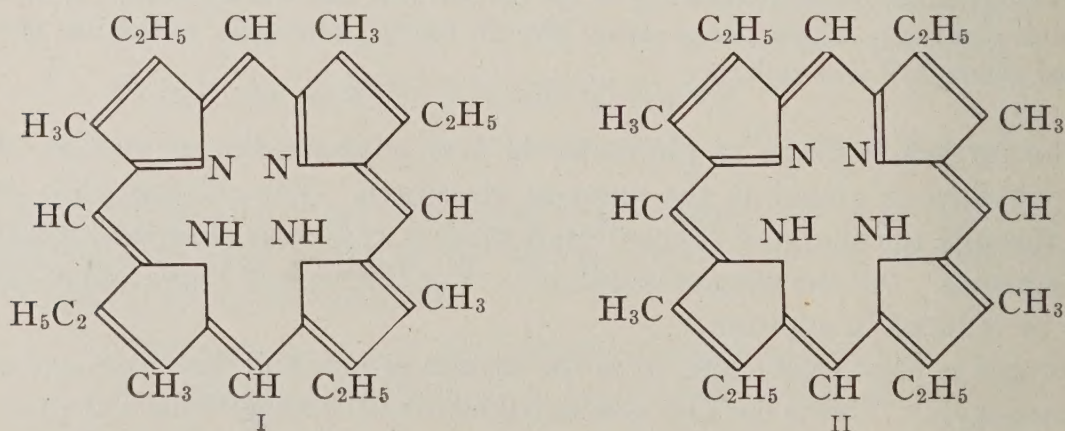


Phylloporphyrin can be prepared from phytochlorin by treatment with alkali at 150° or from phytorhodin by means of alkali and pyridine at 150° .

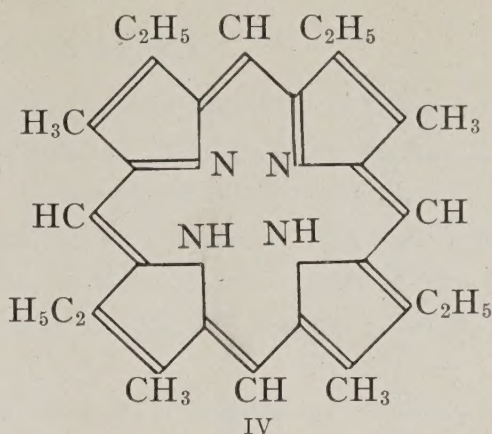
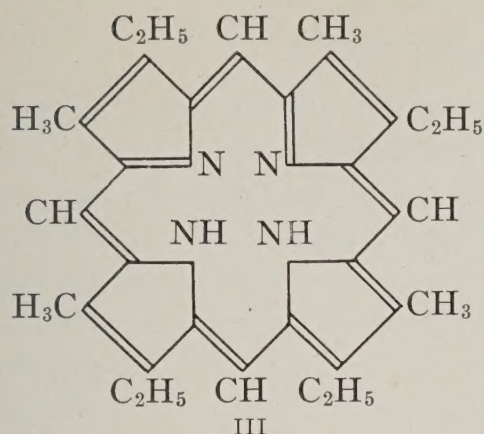
The final carboxyl is removable from phylloporphyrin and from monobasic phyllins only by fusion with soda lime. This gives the parent substances of chlorophyll. From the phyllins comes aetiophyllin and from the porphyrins comes aetioporphyrin:

Aetiophyllin and aetioporphyrin are easily interchanged by the usual methods. Aetiophyllin contains 8% magnesium, is easily dissolved in organic solvents and still has the color and fluorescence of chlorophyll. Aetioporphyrin has a bronze color and reacts with heavy metals to form characteristic complex compounds.

Through the brilliant syntheses of H. Fischer and his colleagues,¹ it has been demonstrated that aetioporphyrin has the composition $\text{C}_{32}\text{H}_{38}\text{N}_4$ and represents the union of four pyrroles, each of which bears one methyl and one ethyl group. Depending on the position of the side chains, there are four possible isomers, all of which have been prepared synthetically:



¹ Fischer, H. und G. Stangler. *Liebigs Ann. d. Chem.* 459: 53. 1927; Fischer, H. Friedrich, W. Lamatsch und K. Morgenroth. *Ibid.* 466: 147. 1928; Fischer und K. Zelle. *Ibid.* 468: 98. 1929; Fischer, K. Platz und K. Morgenroth. *Zeitschr. f. physiol. Chem.* 182: 265. 1929; and many other papers. Summary: Fischer, H. *Handbuch d. normal. und path. Physiologie.* 6: 164. 1928.



Formula III corresponds to the blood pigment. The constitution of aetioporphyrin prepared from chlorophyll remains to be determined. In any case, the preparation of the basic substances allows us to discuss the constitution of the immediate derivatives of both chlorophyll and the pigment of the blood.

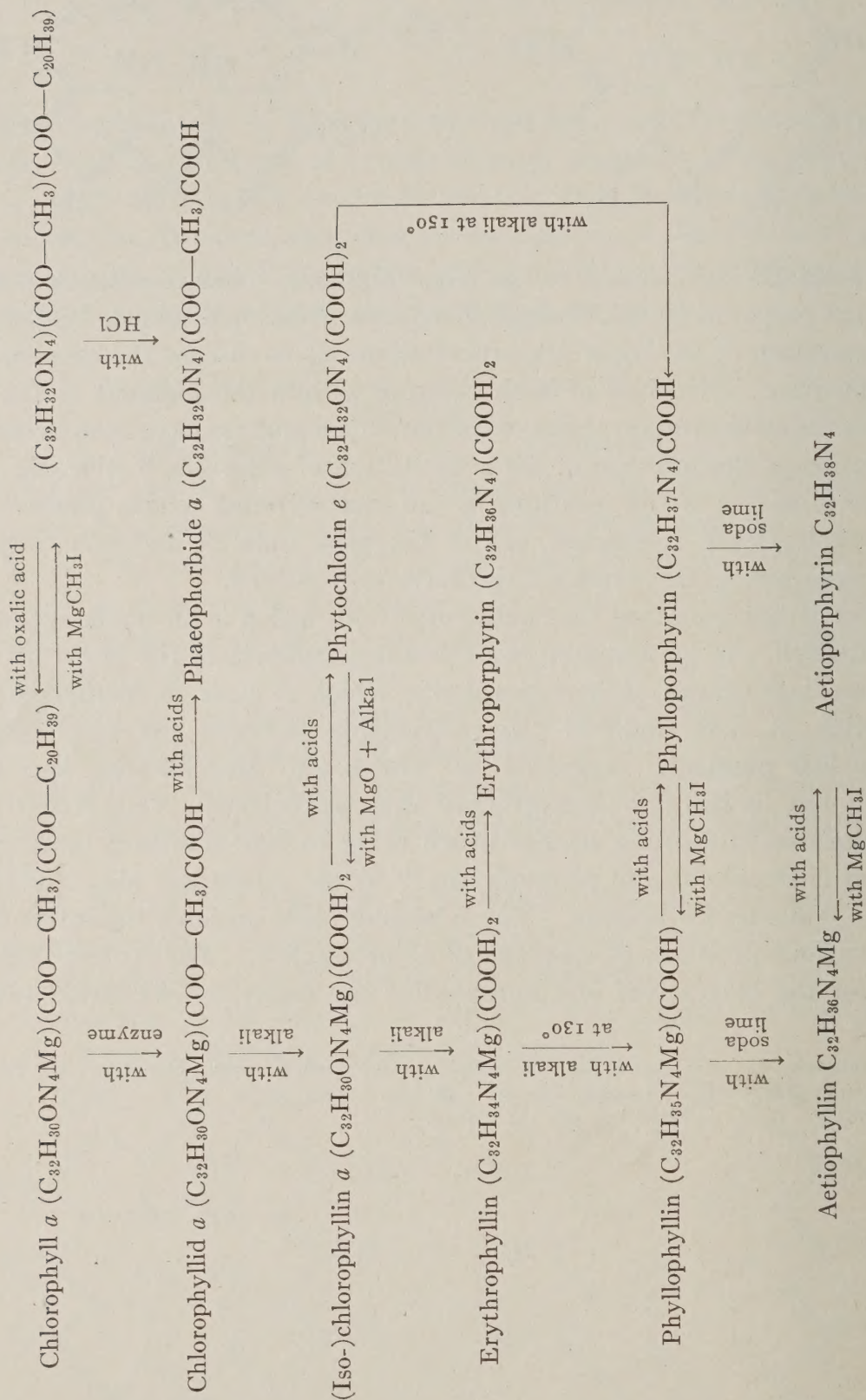
After the structural formulae of the parent substances have been determined, the solution of the problem of the constitution of chlorophyll will only require the determination of the positions of the carboxyl and lactam groups. After this work has been completed, one of the most difficult and most important problems of biological chemistry will have been solved.

Hence it has appeared that aetioporphyrin, which is an unchanged residue of chlorophyll, can be prepared from hematoporphyrin of the blood by way of the immediate stages of hemoporphyrin which is close to erythroporphyrin. Willstätter at first doubted the relationship between the blood pigments and the leaf pigments, suggested by Schenk and Marchlewsky and also by Nencki,¹ but he has now strengthened this supposition. Very recently there have appeared interesting studies which bring to light a strong blood-forming action of chlorophyll² and phaeophytin.³ These substances also appear to be effective remedies for anemia. The following table explains the inter-relations of the various products of cleavage of chlorophyll *a*. The corresponding formulae for the derivatives of chlorophyll *b* can easily be calculated from this table.

¹ Marchlewsky, L. Die Chemie des Chlorophylls. 1909; Nencki. Ber. chem. Ges. 29: 2877. 1896.

² Burgi und Traczewski. Biochem. Zeitschr. 98: 256. 1919.

³ Grigoriew. Biochem. Zeitschr. 98: 284. 1919.



6. THE YELLOW LEAF PIGMENTS

Carotin is easily dissolved in organic solvents but especially in benzene and chloroform. It is an unsaturated hydrocarbon, probably in the group of fulvenes. Its empirical formula is $C_{40}H_{56}$.¹ It forms an indigo-blue solution in sulphuric acid. It is quickly oxidized in the air and absorbs up to 40% of its weight of oxygen. Carotin occurs in carrots, in the corpus luteum of the cow² and in the blood of animals which have colored fats.³ Isomeric with it is *lycopin*, $C_{40}H_{56}$, the pigment of tomatoes.⁴

Xanthophyll is insoluble in petrol ether and is therefore easily separated from carotin. The composition of xanthophyll is $C_{40}H_{56}O_2$,⁵ so it probably has

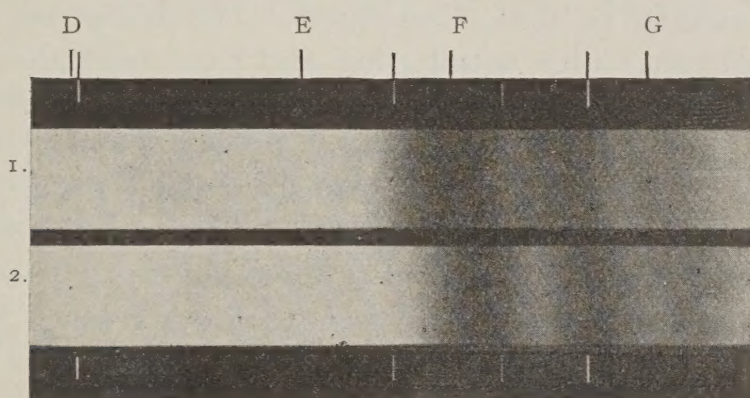


FIG. 10.—1. Absorption spectrum of carotin. 2. Absorption spectrum of xanthophyll. (After Willstätter and Stoll.)

the same structure as carotin. It also absorbs atmospheric oxygen. *Lutein*, the pigment of egg yolk has the same composition and the same properties except a different melting point.⁶

In the absorption spectra of carotin and xanthophyll (Fig. 10) there are two remarkable absorption bands in the blue-violet part of the spectrum. They are much better defined than the corresponding absorption bands of chlorophyll, and overlap in part. For that reason the absorption of the blue-violet rays by chlorophyll is much reduced.⁷

The brown algae contain another interesting pigment—fucoxanthine—which from solution in methyl alcohol forms crystals of brownish-red prisms. It has well defined basic properties and does not absorb atmospheric oxygen. The action of alkali changes it to pyrone and it forms a labile union with the alkali. The empirical formula of fucoxanthine $C_{40}H_{54}O_6$ indicates its relationship to carotin and xanthophyll. Like them it has no fluorescence.

¹ Willstätter und Mieg. *Liebigs Ann. d. Chem.* 335: 1. 1907.

² Escher, H. H. *Zeitschr. f. physikal. Chem.* 83: 198. 1913.

³ Palmer, L. S. *Jour. Biol. Chem.* 27: 27. 1916.

⁴ Willstätter und Escher. *Zeitschr. f. physikal. Chem.* 64: 47. 1910. For other carotinoids cf. Kylin. *Zeitschr. f. physiol. Chem.* 157: 148. 1926; 168: 229. 1927.

⁵ Willstätter und Mieg. *loc. cit.*

⁶ Willstätter und Escher. *Zeitschr. f. physiol. Chem.* 76: 214. 1912.

⁷ Iwanowsky, D. The question of the second assimilation maximum. 1915 (Russian).

7. EXTRACTION AND QUANTITATIVE DETERMINATION OF LEAF PIGMENTS

To extract chlorophyll from leaves, the usual practice is to use carefully dried leaves. The best method is the new one of Willstätter and Stoll.¹ Finely divided dry or fresh leaves are placed on a suction filter under a weak suction and treated with 85% acetone in water.² Willstätter found that almost all the pigments went into solution very quickly. The chlorophyll is then transferred to petrol ether by Kraus' method. This solution is first shaken several times with acetone to remove the foreign matter and then with water to remove the acetone. The xanthophyll is removed by transferring it to 80% methyl alcohol and the chlorophyll is freed from carotin by shaking the petrol ether with water in order to remove the last traces of acetone and all of the methyl alcohol (chlorophyll is insoluble in pure petrol ether). The precipitate of chlorophyll is washed several times with petrol ether and freed from the last traces of carotin by repeated solution in ether and precipitation in petrol ether. This procedure gives a pure yield of 75% of the total chlorophyll content of leaves. The separation of the components *a* and *b*³ is effected by transferring the chlorophyll *a* to petrol ether saturated with methyl alcohol and the chlorophyll *b* to 90% methyl alcohol (by Kraus' method of separation).

The yellow pigments are separated from each other when the chlorophyll is being prepared.⁴ After transferring the chlorophyll from the acetone extract to petrol ether, all the xanthophyll is removed with methyl alcohol (see above). By diluting the methyl alcohol with water and treating the solution with ether, the xanthophyll is transferred to the ether. Then this solution is shaken with alcoholic lye until the chlorophyll impurity is saponified and the chlorophyllin can be washed out with water. The final purification of xanthophyll is done by crystallization from methyl alcohol. The carotin remains in solution after the chlorophyll is precipitated from the pure petrol ether (see above). The filtrate is concentrated in a vacuum at 40° and treated with 90% alcohol. The pure carotin will at once crystallize out.

The quantitative determination of the leaf pigments is done colorimetrically.⁵ In the case of comparative experiments with different plants it is advisable to change the chlorophyll to chlorophyllin and separate out the yellow pigments. Duboscq's colorimeter is recommended for quantitative work but the spectrophotometer is better and more accurate.⁶ If it is necessary to obtain absolute measurements, the solutions to be studied can be compared with suitable standard solutions. The spectrophotometric method cannot be used with solutions for comparisons.

¹ Willstätter und Stoll. Untersuchungen über Chlorophyll. P. 132. 1913 [(translated by Schertz in 1928); among the several modifications of this method, those mentioned by Schertz (Plant Physiol. 3: 211-216. 1928) have been found reliable and convenient].

² The use of alcohol is not recommended on account of the allomerization in alcoholic solution.

³ Willstätter und Stoll. *Ibid.* P. 161.

⁴ Willstätter und Stoll. *Ibid.* P. 237.

⁵ Willstätter und Stoll. *Ibid.* P. 78; [improved colorimetric methods are given by Schertz (Plant Physiol. 3: 323-334. 1928) who also gives the essentials of the spectrometric methods which are just coming to be used for the most accurate work].

⁶ Monteverde and Lubimenko. Mitt. d. russ. akad. d. Wiss. 1913 (Russian); Weigert, F. Ber. chem. Ges. 49: 1496. 1916.

In order to determine the single components a and b , they are first changed to phytochlorin and phytorhodin and the latter are determined colorimetrically. The yellow pigments are separated from each other and measured alone. Comparison solutions are prepared for phytochlorin, phytorhodin and each of the yellow pigments. In this way it is possible to make quantitative determinations of all the leaf pigments and to measure their proportions in different plants.

8. THE RATIO OF THE SINGLE PIGMENTS IN DIFFERENT PLANTS

Countless determinations of the green pigments have shown that in the majority of seed-plants there are 3 molecules of chlorophyll a to 1 of chlorophyll b . The ratio is constant day and night and so does not depend on photosynthesis.¹

There is usually more chlorophyll b in shade leaves. The ratio of the yellow pigments averages 1 molecule of carotin to about 2 of xanthophyll. The amount of green pigments in the leaf is considerably greater than that of the yellow ones, the ratio being 1 molecule of the yellow pigments to 3-4½ of the green pigments. All these ratios are shown in the following table from Willstätter and Stoll. $Q\frac{a}{b}$ stands for the ratio between chlorophyll a and b , $Q\frac{c}{x}$ the ratio between carotin and xanthophyll and $Q\frac{a+b}{c+x}$ the ratio between the green and yellow pigments.

Plants		$Q\frac{a}{b}$	$Q\frac{c}{x}$	$Q\frac{a+b}{c+x}$
Shade leaves	<i>Sambucus nigra</i>	2.74	0.588	3.33
	<i>Sambucus nigra</i>	2.83	0.629	2.83
	<i>Sambucus nigra</i>	2.90	0.510	3.10
	<i>Aesculus hippocastanum</i>	2.89	0.699	2.84
	<i>Platanus acerifolia</i>	3.52	0.478	3.98
	<i>Fagus sylvatica</i>	3.13	0.653	3.45
Sun leaves	<i>Sambucus nigra</i>	2.07	0.345	4.63
	<i>Aesculus hippocastanum</i>	2.30	0.353	4.70
	<i>Platanus acerifolia</i>	3.16	0.433	3.31
	<i>Fagus sylvatica</i>	2.92	0.553	6.02

The chlorophyll content per unit weight of leaves undergoes marked fluctuations even for different leaves on the same plant. In general, chlorophyll constitutes from 0.6 to 1.2% of the dry weight of leaves and its content does not vary for the different hours of the day. It is difficult to compare the chlorophyll content of different plants because of the inaccuracy of the calculations. Shade leaves contain more chlorophyll than sun leaves if calculated on the basis of dry weights but if reckoned per unit of surface the reverse ratio

¹ Willstätter und Stoll. loc. cit. P. 109.

holds.¹ For plants of different geographical ranges, the chlorophyll content per unit of weight remains fairly constant. For plants of sunny habitats it usually falls and for shade plants it increases in passing from pole toward the equator.² By varying the supply of nitrogen, it is found that the chlorophyll content increases with favorable doses of this element.³

In the green algae the ratio of the pigments is almost the same as that in seed-plants but the brown algae contain a larger amount of the yellow pigments which also affects the color of these plants. For example, the following molecular ratios have been found for *Fucus*, *Dictyota* and *Laminaria*:

Q	Chlorophyll	Ratio of			
	Carotin, Xanthophyll & Fucoxanthine	Carotin: Xanthophyll: Fucoxanthine			
<i>Fucus</i>	0.95	1.08	:	1	: 1.75
<i>Dictyota</i>	1.20	0.77	:	1	: 3.60
<i>Laminaria</i>	1.07	0.16	:	1	: 1.92

In the brown algae the chlorophyll is almost entirely chlorophyll *a*.⁴ Only chlorophyll *a* is present in the saprophytic *Neottia* which is very rich in yellow pigments.⁵ The statements of some investigators to the effect that brown algae contain a pigment *phaeophyll* which readily changes to chlorophyll⁶ have been contradicted by more recent studies.⁷

9. FORMATION OF CHLOROPHYLL IN PLANTS

Unfortunately this important process has been studied very little from the chemical side. This is the more to be regretted because such studies might help to explain the physiological rôle of chlorophyll.

It is certain that the formation of the green pigments represents a photochemical process in most seed-plants. Seedlings grown in darkness remain pale yellow and contain no chlorophyll although they have a certain amount of the yellow pigments. Such plants are said to be etiolated. The necessity of light for the greening of plants was known even to Aristotle but this rule does not apply to all plants. Many needle-bearing trees also form chlorophyll in the dark but in smaller amounts than in the light.⁸ Moreover greening in darkness has been observed in some ferns,⁹ mosses¹⁰ and algae.¹¹ In the Angiosperms such cases are limited to *Pistacia*, *Eryobotria* and a few other plants.¹²

¹ Willstätter und Stoll. *loc. cit.* P. 114.

² Lubimenko, W. The transformations of the plastid pigments in living plant tissues. 1916 (Russian). However, cf. Henrici, M. Dept. of Agr. Union of S. Afr. 11/12: 259. 1927.

³ Wille. Compt. rend. 109: 397. 1889; Boresch, K. Jahrb. Wiss. Bot. 52: 145. 1913; Magnus und Schindler. Ber. bot. Ges. 30: 314. 1912.

⁴ Willstätter und Page. Liebigs Ann. d. Chem. 404: 237. 1914.

⁵ Wilschke. Zeitschr. f. wiss. Mikroskopie. Vol. 31. 1914.

⁶ Molisch, H. Bot. Zeit. 63: 131. 1905.

⁷ Tswett, M. Ber. bot. Ges. 24: 235. 1906; Idem. Chromophyll etc. (Russian); Kylin, H. Zeitschr. f. physiol. Chem. 82: 221. 1912; Willstätter und Page. *loc. cit.*

⁸ Sachs, J. Flora, 1862, p. 186; 1864, p. 505; Wiesner, J. Die Entstehung des Chlorophylls. P. 117. 1877; Burgerstein, A. Ber. bot. Ges. 18: 168. 1900.

⁹ Schimper. Jahrb. wiss. Bot. 16: 159. 1885; Bittner, K. Österr. bot. Ges. 1905, p. 302.

¹⁰ Schimper. *loc. cit.*; Bittner. *loc. cit.*; Treboux. Ber. bot. Ges. 22: 572. 1904.

¹¹ Heinricher, E. Ber. bot. Ges. 1: 442. 1883; Schimper. *loc. cit.*; Artari. Ber. bot. Ges. 19: 7. 1901.

¹² Lopriore. Ber. bot. Ges. 22: 385. 1904; Ernst. Beitr. z. bot. Centralbl. 19: 118. 1905; Atwell. Bot. Gaz. 15: 46. 1890.

According to the careful studies of Liro,¹ the action of light is only the final process in a series of reactions. This process changes the colorless chromogen—the so-called *leucophyll*—into the colored chlorophyll. This is a purely chemical reaction which proceeds in killed and macerated plant tissues from which oxygen is excluded. The strongest effects come from the rays absorbed by chlorophyll, especially the red rays between B and C, as proved by Weisner.²

Leucophyll has not yet been prepared and studied in pure form although such an investigation appears to be possible. Water must be carefully excluded and the chromogen extracted from frozen plants with solvents free from water. The leucophyll is changed in the presence of water, just as in the light and at temperatures above 48°. By the action of light, this changed chlorophyll does not become chlorophyll but another green pigment called *protochlorophyll*.³ This has a characteristic absorption spectrum in which the most important first absorption band of chlorophyll (between B and C) is lacking, although the second band in the orange comes out very sharply ($\lambda = 640-620\mu\mu$; see Fig. 8). This substance has also not been investigated chemically and it is an open question as to its difference from chlorophyll. On the basis of spectroscopic studies, Noack and Kiepling⁴ conclude that protochlorophyll is not a secondary product but a preliminary step to the formation of chlorophyll. Protochlorophyll contains magnesium and is said to be changed to chlorophyll by photo-oxidation. Of course, spectroscopic observations may not settle the question conclusively (*cf.* pp. 74 and 77).

Aside from light, several other conditions are necessary for the synthesis of chlorophyll. The work of Liro (*loc. cit.*) shows, however, that all these conditions are related to the formation of leucophyll which can take place in darkness. The need of iron is especially striking⁵ although this metal is not a part of chlorophyll. The iron probably accumulates in large amounts in the colorless stroma of the chloroplasts⁶ and acts as a catalyst to the oxidation processes which take place in the synthesis of leucophyll. However, it is more likely that iron participates directly in the photosynthesis.⁷ A certain amount of oxygen is also necessary.

If iron is lacking the plants remain yellow but differ sharply from etiolated plants in their normal form of plant body. This plant disease is called *chlorosis* and is remedied by the addition of iron salts. It must be pointed out that chlorosis also appears when various other mineral substances are lacking.⁸

¹ Liro, J. Über photochemische Chlorophyllbildung. 1908; *cf.* also Issatschenko. Ber. bot. Gart. 9: 105. 1909 (Russian).

² Wiesner. *loc. cit.*; Schmidt. Cohns Beitr. z. Biol. d. Pflanzen. 12: 269. 1914.

³ Monteverde. Acta Horti. Petropolit. 13: 201. 1894; *Idem.* Ber. d. bot. Gart. in Petersburg. 9: 105. 1909 (Russian); Liro. *loc. cit.*; Monteverde und Lubimenko. Ber. d. russ. Akad. d. Wiss. 1911, p. 73; 1912, p. 609 (Russian). [See also Eyster, W. H. Science. 68: 569-570. 1928.]

⁴ Noack, K. und W. Kiepling. Zeitschr. f. physiol. Chem. 182: 13. 1929. [See also Noack, K. Forschungen und Fortschritte. 5: 100-101. 1929; Schertz. Science. 70: 258-259. 1929.]

⁵ Gris, E. De l'action des comp. ferrug. sur la vegetation. 1843; Compt. rend. 19: 1110. 1844; Molisch, H. Die Pflanzen in ihren Beziehungen zum Eisen. 1892.

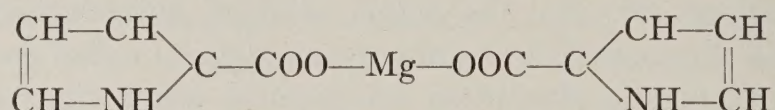
⁶ Moore, B. Proc. Roy. Soc. London (B) 87: 556. 1914.

⁷ Wurmser, R. Recherches sur l'assimilation chlorophyllienne. 1921.

⁸ Gris. *loc. cit.*; Wille. *loc. cit.*; Roux. Traité des rapports des plantes avec le sol. 1900; Sidorin. Resultaten der Vegetationsversuche und Laboratoriumsarbeiten v. D. Prjanischnikow. 10: 241. 1914.

Still iron has more effect than the other elements. It is interesting that an *excess of calcium* can bring about chlorosis in plants unaccustomed to lime soils.¹ The author of this book has observed a chlorosis of fruit trees on soils poor in lime when watered too much with irrigation water containing large amounts of lime. Chloroses induced by manganese have also been described,² since the physiological effect of manganese is said to be antagonistic to that of iron. On the other hand, there are references in the literature³ to chlorosis brought about by a complete absence of manganese. These results need to be confirmed.

The latest results indicate that iron is necessary only for the synthesis of the following intermediate product:



If this salt of pyrrol- α -carboxylic acid is supplied to plants, they are said to turn green in the complete absence of iron.⁴ This question requires further investigation.

Of course, plants turn green only if they are provided with sufficient amounts of the materials to form chlorophyll, *i.e.*, magnesium salts and organic nutrients, both with and without nitrogen.⁵ Leucophyll is not synthesized in the absence of oxygen nor at temperatures so low or so high that the normal activity of the plants is disturbed.⁶

10. THE RATIO OF ENERGY ABSORBED TO WORK PERFORMED IN LEAVES

Ingen-Housz, de Saussure and other pioneers in the field of photosynthesis were unable to prove exactly that the solar energy is actually the direct driving force of physiological processes in plants. The science of that time was not equal to such a demonstration. It proved that the assimilation of carbon and the other elements which are necessary for the synthesis of organic compounds, did not occur except under the influence of the sun's rays and so it appeared unnecessary and scarcely of interest to take up the quantitative side of the problem. Later this question came to be connected with the development of thermodynamics and its solution proved to be very necessary. On the other hand, advances in physiology taught that irritant phenomena were important in organisms. It was first necessary to compare these phenomena with the processes which release energy. This analogy must have been suggested with the same reserve as that between a living organism and a chemical factory

¹ Maze, Ruot et Lemoigne. Compt. rend. 155: 435. 1912; Büsgen. Bot. Jahrb. 50 (Suppl.): 526. 1914; Robert. Bull. Soc. de Biol. 1: 84. 1914; Masoni. Stat. sperim. agr. ital. 47: 647. 1914; Canda. Ibid. 47: 627. 1914; de Angelis d'Ossat. Ibid. 47: 603. 1914; Creydt. Jour. f. Landwirtschaft. 63: 125. 1915; Pfeiffer und Simmermacher. Landwirt. Versuchs-Stat. 93: 1. 1919.

² Tottingham and Black. Plant World. 19: 359. 1916; Johnson. J. Ind. Eng. Chem. 9: 47. 1917; Rippel, A. Biochem. Zeitschr. 140: 315. 1923.

³ McHargues. Jour. Amer. Chem. Soc. 44: 1592. 1922.

⁴ Oddo, B. Gazz. Chim. Ital. 50: 54. 1920; Pollacci. Ibid. 45(2): 197. 1915. Cf. also Godnew, T. N. und N. A. Naryschkin. Ber. chem. Ges. 58: 2703. 1925.

⁵ Palladin, W. Ber. bot. Ges. 9: 229. 1891; Idem. Rev. gén. bot. 9: 385. 1897.

⁶ Wiesner, J. Sitzungsber. d. Akad. Wien, Math.-naturw. Kl. I. 69: 327. 1874; Idem. Entstehung des Chlorophylls. 1877.

or a coördinated system of apparatus. In any case, there is a liberation of energy stored in the organism in the case of all irritant phenomena but the stimulation is usually the result of the action of external factors on the organism. It is clear that there is no ratio between the energy used for the stimulus and the work done as a result of the stimulation. This was discussed in Chap. I (*cf.* pp. 30-31).

As we shall see later, light is often a stimulus to plants and in all these cases the amount of light energy absorbed by the plant does not correspond in any way to the work done. To return to photosynthesis, it is evident that when we postulate the use of solar energy for the nourishment of all life on earth, we must prove that the basic principles of photochemistry apply to photosynthesis. Only when it is proved that these principles have unlimited control of the nutrition of plants through light, do we have the right to assume the direct use of solar energy and its transformation into chemical energy.

Accordingly, we must prove that the three principles of photochemistry stated on pages 67-68 apply to photosynthesis. The first law states that only absorbed rays exert a chemical effect; according to the second law, the photochemical output is directly proportional to the amount of energy absorbed; the third law says that a constant fraction of the energy absorbed is used to do chemical work.

II. ANALYTICAL METHODS

For the qualitative determination of the absorbed energy, rays of definite wave lengths must be isolated. This is done in two ways—either by using light filters or by breaking up the solar spectrum with a prism. In the first case use is made of colored glass or liquids and it is essential to use a spectroscope for a control because it must be determined which rays are transmitted by these filters. The best optical firms now furnish glass which transmits only visible waves of definite wave lengths. If a leaf is exposed to the light filtered through such a glass it is certain that the other rays do not strike the leaf. Glass which is not specially made for light filters must be used with the utmost care.

Colored liquids are usually placed in double-walled glass bell jars (Fig. 11) which were first used by Senebier. The plants or parts of plants to be studied are placed under the bell jar. The following two liquids are recommended as light filters; (1) a solution of potassium dichromate which transmits only red, orange, yellow and part of the green rays if the concentration is not too high, and (2) an ammoniacal solution of cupric oxide which transmits the rest of the green, the blue and the violet rays but absorbs all the red, orange and yellow rays. It is evident that the rays which are transmitted by these liquids are always weakened according to the concentration of the solution and the thickness of the layer of liquid.

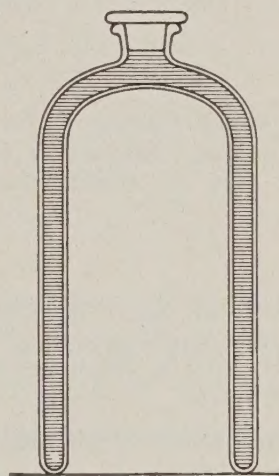


FIG. 11.—Senebier's bell jar. The space between the double walls of the bell jar is filled from the top with a colored liquid. (*After Sachs.*)

The sunlight must be broken up to obtain more exact removal of the single rays. A beam of sunlight from a mirror is passed into a dark chamber through a narrow slit. The beam is broken up in a prism and reflected as a spectrum on a white screen. The leaves to be studied are exposed at various zones of the spectrum. This method makes it possible to isolate completely the rays absorbed by chlorophyll from those not absorbed.¹

For more exact quantitative determinations it must be borne in mind that different light filters do not have an equal effect in reducing the intensity of the light which they transmit so that the ratios of their intensities are not the same as in white light. It is not an easy task to adjust the concentration of the liquids so that, for example, the rays transmitted by the solution of potassium dichromate are decreased in intensity exactly as much as those which pass through the solution of ammoniacal copper oxide.² If the work is being done in a spectrum, it is necessary to allow for the error introduced by the unequal dispersion

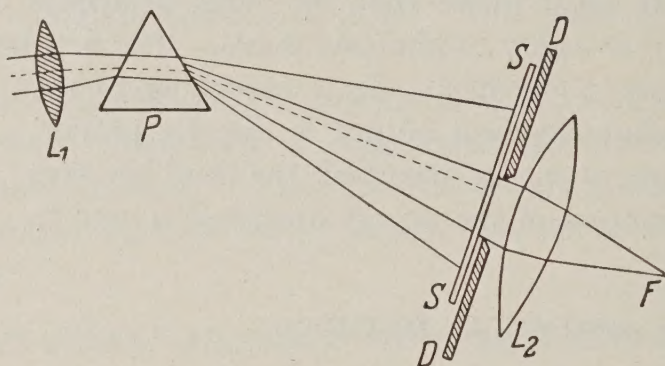


FIG. 12.—“Spektrophor” (diagrammatic). L_1 , lens; P , prism; SS , scale of wave lengths; DD , diaphragm to screen out certain rays; L_2 , large concentrating lens; F , objective image of the sun in which the experimental object is placed. (After Reinke.)

of the more strongly and less strongly refrangible rays in the prismatic spectrum. A diffraction spectrum cannot be used because it is not bright enough. This unequal dispersion is excluded by the ingenious apparatus designed by Reinke and called a “spektrophor.”³ The principle of the apparatus may be seen from Fig. 12. The beam is broken up in the prism P and falls as a spectrum on a ground glass provided with a scale SS . Behind this there are two movable pieces of wood DD , by the aid of which any desired part of the spectrum may be passed on and all the other rays screened out. The lens L_2 collects the transmitted rays into an objective image of the sun at F ; this removes the inequality of the dispersion. The object to be studied is placed in the solar image.

The quantitative determination of the energy absorbed can be carried out by means of different apparatus, the descriptions and directions for use of which may be found in books on general physics. General suggestions are sufficient for our purpose here. Photometric methods give only approximate values. The brightness of the light on the upper and lower surfaces of the leaves is

¹ For further details of the method cf. Timiriazeff, K. The assimilation of light by plants. 1875 (Russian).

² Richter, A. v. Rev. gén. bot. 14: 151. 1902.

³ Reinke, J. Bot. Zeit. 42: 1. 1884.

missed. However, the intensity of the light can never serve as an objective measure and such determinations are inexact. The use of the radiometer¹ gives better results but the greatest accuracy comes from measuring the absorbed energy by the aid of a Rubens thermopile or, better still, a bolometer.² The quantities of energy used in photosynthesis can be measured especially well by these devices. As it will be explained later, such determinations are made with great difficulties. Moreover, the use of exact methods of measuring energy requires the use of equally good methods of determining the chemical work performed, and vice versa. Unfortunately this uniformity is not always attained and the reliability of the results is thereby diminished.

The qualitative demonstration of photosynthesis is usually made from the liberation of oxygen. A very popular method is to follow the release of bubbles

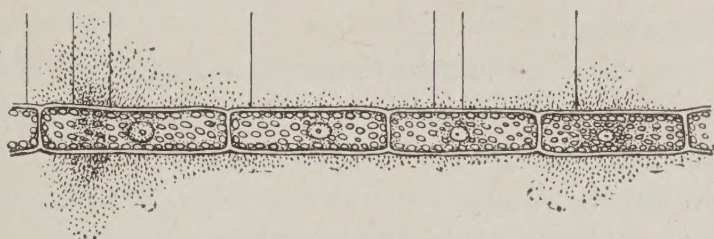


FIG. 13.—The use of Engelmann's bacterial method. Bacteria which are motile only in the presence of oxygen collect on the filament of green alga on which a prismatic spectrum is projected. The bacteria collect only in the zones of the spectrum in which the light is strongly absorbed by chlorophyll. (After Engelmann.)

of gas from water plants placed in the light; it is easy to demonstrate a high content of oxygen in the gas thus liberated. Boussingault (see p. 65) used the luminescence of phosphorus in darkness to prove the presence of oxygen. Oftentimes use is made of the return of the normal color of pigments bleached with hydrosulphite.³ The most sensitive reactants to oxygen are aerobic bacteria. The excellent bacterial method worked out by Engelmann⁴ is invaluable because it can be used for microscopic observations, as in studies of the localization of photosynthesis in definite parts of cells. Some bacteria, such as *Bacterium termo*, are motile only in the presence of oxygen. If a green alga or a living portion of a green plant is mounted in a drop of water on a slide, to it added a pure culture of bacteria sensitive to oxygen, the drop covered with a cover glass and the edges sealed with vaseline, the first observations in a darkened room will find the bacteria still motile but after the oxygen is consumed they will come to rest and remain motionless. If the preparation is now returned to the light so that the alga starts to liberate oxygen, the bacteria will again become active and will collect in a motile condition at the source of oxygen (see Fig. 13). This method makes it possible to demonstrate the slightest

¹ Brown, H. T. and F. Escombe. Proc. Roy. Soc. London (B) 76: 39. 1905.

² Puriewitsch, K. Jahrb. wiss. Bot. 53: 210. 1913; Warburg, O. und E. Negelein. Zeitschr. f. Elektrochem. 102: 235. 1922; 106: 191. 1923.

³ Kny. Ber. bot. Ges. 15: 388. 1897.

⁴ Engelmann. Pflügers Arch. f. ges. Physiol. 57: 375. 1894; Idem. Bot. Zeit., 39: 442. 1881; 40: 419 and 663. 1882; 41: 1. 1883; 44: 43. 1886; 45: 100. 1887.

traces of oxygen in the liquid. Another delicate method is to use luminescent bacteria which require oxygen to emit light.¹



FIG. 14.—Exposure of leaf for a measurement of photosynthesis. The leaf is inclosed in a glass tube with flat sides and a closed upper end and the inside atmosphere is enriched in CO_2 .

Unfortunately, all these ingenious and sensitive qualitative tests are often used for quantitative measurements. This is inadmissible. The method of counting gas bubbles has been used very frequently in quantitative experiments. In the first place, the oxygen content of the bubbles is unknown; secondly, the diameters of the bubbles are not constant; and thirdly, the rate at which the bubbles are released depends on a number of variable factors such as the nature of the cut surface.² It is certain that such unreliable quantitative methods have been used in no other field of plant physiology. The bacterial method has often been used for quantitative studies but it is just as bad.

A correct quantitative determination of photosynthesis can be made either on the basis of the gas exchange or by measuring the increase in dry weight of the leaf. If study of the rate of the process under natural conditions is to be disregarded and it is desired to perform the experiment in the shortest possible time (in order to avoid deterioration of the material and change to abnormal conditions) the gasometric method is to be recommended. The leaf to be studied is placed in a measuring tube with flat sides (a round glass tube may produce a dangerous convergence of the direct rays of light) and sealed with mercury after increasing the per cent of carbon dioxide in the tube (Fig. 14). The leaf is exposed to the sun or artificial light. Previous to the exposure, the content of CO_2 and O_2 in the mixture of gases about the leaf must be determined by analysis. The total volume of gas in the tube must also be known. The gas analysis is repeated after the exposure to light in order to measure the decrease in CO_2 and the increase in O_2 . The rate of photosynthesis is expressed by the amount of CO_2 used per hour per unit of leaf area.² The gas analysis is to be carried out by one of the modern exact methods which are described in appropriate books.³ The same books will also give directions for measuring the total volume of gas. The exact apparatus

¹ Beijerinck. *Centralbl. f. Bakteriöl.* (2) 9: 685. 1902; Molisch, H. *Bot. Zeit.* 62: 1. 1904; Richter, A. v. und Kollegorskaja. *Mitt. d. russ. Akad. d. Wiss.* 1915, p. 457 (Russian); [Harvey and Morrison (*J. Gen. Physiol.* 6: 13. 1924) used methods developed by the former and his colleagues and found that they were able to detect 1 part of O_2 in 3,700,000,000 parts of sea water].

² For a thorough study of the several factors which enter into such experiments, see Kneip, H. *Jahrb. wiss. Bot.* 56: 460-510. 1915.—Ed.

³ Kostytschew, S. *Jour. russ. bot. Ges.* 5: 50. 1920 (Russian). Strictly speaking, the rate should be calculated according to the *surfaces* of the active chloroplasts; cf. Briggs, G. E. *Proc. Roy. Soc. London (B)* 94: 20. 1922.

⁴ Bunsen, R. *Gasometrische Methoden.* 1877; Hempel. *Gasanalytische Methoden.* 1913. [Reliable manuals in English include: Haldane, J. S. *Methods of Air Analysis.* London. 1912; Hempel, W. *Methods of Gas Analysis.* Transl. by L. M. Dennis. London. 1892. See Spoehr, H. A. *Photosynthese.* (1926) P. 245 ff. for a resume of the best methods.]

of Doyère and of Polowzow-Richter are especially good.¹ The latter is described in Chap. VIII. Less accurate results are obtained with the apparatus designed by Bonnier and Mangin² but in many cases this is sufficiently accurate.⁹ The apparatus is so simple that it is useful for field trips and long expeditions. The leaf area is determined either by tracing the leaf on a sheet of paper or by taking an impression on sensitized paper, after which it can be obtained by means of a planimeter or by weighing the tracings and computing the area on the basis of the weight of a unit area of the same paper.³ It is also permissible to cut out a certain area of leaf in advance because mutilation of the leaf has no effect on the rate of photosynthesis.⁴ In conclusion, it may be mentioned that there should be no impurities in the CO₂ added to the tube; it is best to prepare the CO₂ by heating chemically pure sodium bicarbonate.

The convenient and exact method of gas analysis cannot be used for determinations of photosynthesis under natural conditions because the normal percentage of carbon dioxide in the atmosphere does not exceed 0.03% and increasing this concentration results in a considerable increase in the rate in comparison with natural conditions. Sachs⁵ proposed his half-leaf method for experiments under natural conditions. A certain number of leaves of the plants to be studied are divided into halves at the midribs. An accurately measured area (about 500 cm²) of these half-leaves is then removed and dried to constant weight. The other halves are left on the plants in the light for several hours or even a whole day; these are then treated like the control lot. The increase in dry weight of the half-leaves left in the light over that of the control halves, calculated per hour per square centimeter of leaf area, serves as a measure of the rate of photosynthesis. The method as described gives only approximate values;⁶ all the later refinements⁷ could not do away with the sources of error peculiar to the principle of the method which is to be recommended chiefly for ecological investigations. The following method is used for exact measurements.^p Large quantities of air (about 50 liters per hour) are drawn through the containers in which the leaf is exposed, and determinations are made of the carbon dioxide absorbed by the leaf. This method was used by Boussingault⁸ and by Kreusler⁹ but was developed especially by H. T. Brown.¹⁰ The great

¹ Doyère. *Ann. de chim. et de physique* (III) 28: 5. 1850; Palladin und Kostytschew. *Handbuch. der biochem. Arbeitsmethoden* by Abderhalden. 3: 490. 1910; Kostytschew. *Pflanzenatmung*, p. 34. 1924. [English edition of the latter transl. by C. J. Lyon. 1927.]

² Aubert. *Rev. gén. bot.* 3: 97. 1891.

⁹ Another simple apparatus which gives fairly accurate results was described by Osterhout. *Amer. Jour. Bot.* 5: 105-111. 1918. It is easily constructed from standard laboratory materials. The substitution of a burette for the large glass tube suggested by Osterhout makes the apparatus even more convenient to use.—*Ed.*

³ Brown, H. T. and Escombe. *Proc. Roy. Soc. London (B)* 76: 38. 1905.

⁴ Kostytschew, S. *Ber. bot. Ges.* 39: 328. 1921. [Ganong, W. F. *Bot. Gaz.* 39: 145-152. 1905.]

⁵ Sachs, J. *Arb. bot. Inst. in Würzburg.* 3: 19. 1883.

⁶ Brown, H. T. and Escombe. *Proc. Roy. Soc. London (B)* 76: 49. 1905.

⁷ Thoday, O. *Ibid.* 82: 1 and 421. 1909. [Another type of modification is the use of opposite leaves in place of half-leaves; cf. Curtis, O. F. *Ann. Bot.* 39: 573-585. 1925; *Amer. Jour. Bot.* 16: 154-168. 1929; Brooks, W. *Inaug. Diss. Halle.* 1892; Denny, F. E. *Amer. Jour. Bot.* 17: 818-841. 1930.]

^p For various other good methods see Spoehr, H. A. *Photosynthesis*. P. 243 ff. 1926.—*Ed.*

⁸ Boussingault. *Agronomie, chim. agric. et physiol.* 4: 267. 1868.

⁹ Kreusler. *Landwirt. Jahrb.* 14: 913. 1885.

¹⁰ Brown, H. T. and Escombe. *Proc. Roy. Soc. London (B)* 76: 29. 1905.

difficulty lies in collecting all the CO_2 from a very rapid current of air (even when large amounts of air pass through the experimental chamber, there may be a lowering of the rate of photosynthesis through shortage of CO_2). This is accomplished by using tall glass tubes, filled with caustic potash solution and provided with devices to increase the surface for absorption. The apparatus usually used for absorption cannot be shown here. Figure 15, which is taken from the well known work of Brown and Escombe [*loc. cit.*] shows the arrangement of some types of apparatus. It is evident that equal volumes of air are simultaneously drawn through the two absorption apparatus. In one

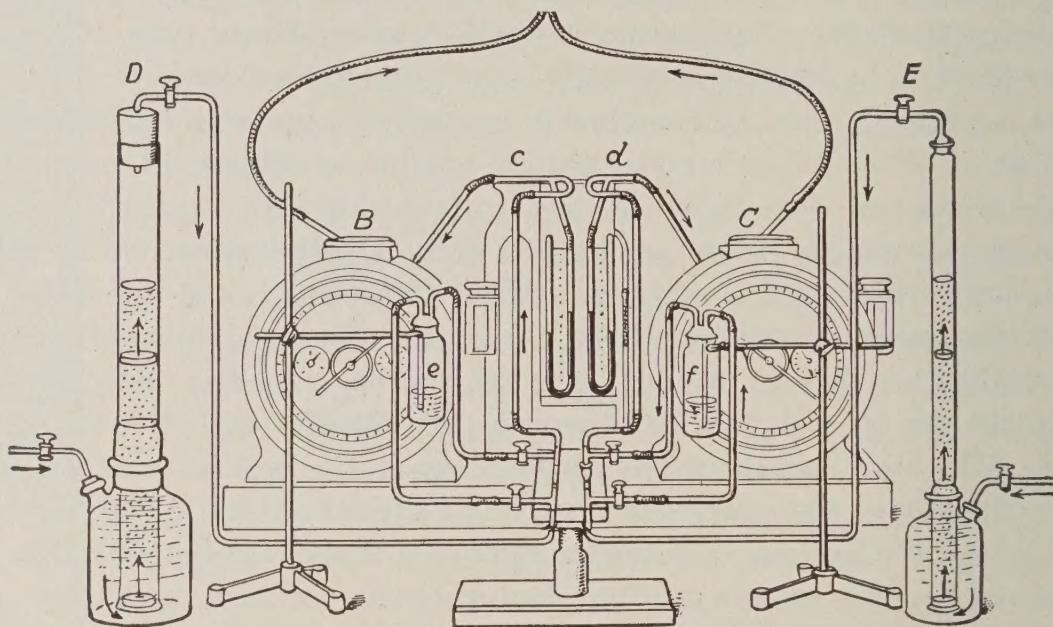


FIG. 15.—The Brown and Escombe apparatus for quantitative determination of photosynthesis in ordinary air. The leaf is inclosed in a chamber not shown here and left in the current of air. CO_2 is absorbed in the receptacle D; c and d are manometers; E is the control vessel of barium hydroxide; B and C are gas meters. Equal volumes of air are passed through the right and left halves of the apparatus, one of which receives air which has not passed by a leaf. Photosynthesis is measured by the difference in the CO_2 caught in the two halves of the system. (After Brown and Escombe.)

apparatus it is necessary to catch all the carbon dioxide of the atmosphere but in the other only the CO_2 which remains in the air after the green leaf in the light has absorbed some of it. The difference between these quantities measures the CO_2 absorbed by the leaf. These experiments are also performed by enriching the air with CO_2 ; in this case the stream of gas can pass more slowly and the CO_2 can be absorbed by the usual apparatus.¹ The results, however, do not express the rate of the process under natural conditions.

The same principle is used in the apparatus of Bazyrina² which seeks to avoid using gas meters and water currents and in this way make it possible to determine photosynthesis in field experiments. The leaf to be studied is not separated from the parent plant in this case.

¹ Blackman, F. F. and G. L. C. Matthaei. *Ibid.* 76: 402. 1905; Matthaei. *Philosoph. Trans. (B)* Vol. 197. 1904; Willstätter und Stoll. *Untersuchungen über die Assimilation der Kohlensäure*, p. 62. 1918; and others.

² Kostytschew, S., K. Bazyrina und W. Tschesnokov. *Planta.* 5: 696. 1928.

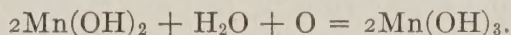
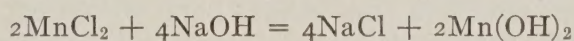
The velocity of the air current in experiments with both these apparatus just described must be at least 1 liter per hour per cm² of leaf surface; otherwise the rate of photosynthesis falls for lack of CO₂.

The apparatus designed by Lundegårdh¹ is built on another principle. An accurately measured quantity of air is drawn into a large metallic bell which is sunk in glycerine by means of a device which is described in the original paper. In the bell, there is a dish of titrated barium hydroxide in which the CO₂ is absorbed. This apparatus can also be used for field experiments. The CO₂-content of the atmosphere is first measured and then that of the air drawn through the leaf chamber.

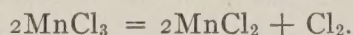
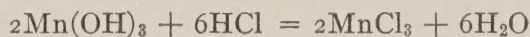
Still other apparatus has been suggested for field experiments.

Recently the gas exchange has often been determined from measurements of change in pressure as recorded by sensitive manometers.² This method makes it possible to obtain a close series of measurements but it involves complicated computations.

For some physiological processes which involve the quantitative determination of photosynthesis, the water plants are better experimental material than land plants because the rate of photosynthesis in the latter often changes unexpectedly by reason of stomata action. To measure the photosynthesis of water plants it is only necessary to measure the amount of oxygen dissolved in the water at the start and again at the close of the experiment, using Winkler's method.³ The method is based on the following reactions. In the presence of alkali, the manganous salt changes to manganic hydroxide at the expense of the dissolved oxygen



Chlorine is liberated on the addition of hydrochloric acid:

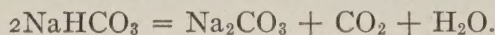


In the presence of potassium iodide, chlorine displaces an equivalent amount of iodine:



The iodine is titrated with thiosulphate. Two atoms of iodine correspond to one atom of oxygen (126 g. iodine = 8 g. oxygen).

In this analysis it is necessary to use enough water to keep all the oxygen in solution after the plants are exposed to light. If the experiment has to be short, the amount of oxygen released may not correspond exactly to the CO₂ absorbed, because these two processes do not take place simultaneously.⁴ For direct determinations of the carbonic acid in the stream of water, Blackman designed a complicated apparatus which is described in the original paper.⁵ Another simple and convenient method consists in adding sodium carbonate to the water and following the change in reaction of the liquid.⁶ The assimilation of CO₂ causes a dissociation of sodium bicarbonate:



¹ Lundegårdh, H. *Kreislauf der Kohlensäure in der Natur*. 1923.

² Barcroft and Hamill. *Jour. of Physiol.* 34: 306. 1906; Warburg, O. *Biochem. Zeitschr.* 100: 230. 1919; 103: 188. 1920.

³ Winkler, L. W. *Ber. chem. Ges.* 21: 2843. 1888; 22: 1764. 1889. [See Osterhout and Haas. *Jour. Biol. Chem.* 32: 141-146. 1917 and Thompson and Miller. *Ind. and Eng. Chem.* 20: 774. 1928 for important modifications.]

⁴ Kostytschew, S. *Ber. bot. Ges.* 39: 319. 1921.

⁵ Blackman, F. F. and A. M. Smith. *Proc. Roy. Soc. London (B)* 83: 374. 1911.

⁶ Osterhout and Haas. *J. Gen. Physiol.* 1: 1. 1918; *Idem.* *Science.* 47: 420. 1918.

If the change in pH is measured by the colorimetric method, using indicators which are non-toxic to the plants, the result is a measure of the absorption of CO_2 in photosynthesis.

Many investigators consider it necessary to correct for respiration in exact experiments. To this end, they first measure the CO_2 liberated in darkness by the leaves used in the experiment. It is assumed that in the light this same amount of CO_2 is developed by respiration and this correction is made in calculating the rate of photosynthesis. Under normal conditions the rate of respiration, even in diffuse light, is only $\frac{1}{10}$ th to $\frac{1}{20}$ th that of the assimilation of CO_2 . However, the introduction of this correction might better be omitted; after leaves are exposed to light their rate of respiration is much greater than before and the rate of respiration during the exposure itself cannot be calculated by a simple interpolation. Hence an incorrect calculation may increase the experimental error through the use of a correction for respiration. Moreover, it often happens that there is a production of CO_2 in the light and the state of the case is thereby still more complicated.¹

12. THE RATIO BETWEEN THE ABSORPTION OF DIFFERENT LIGHT WAVES AND THE ASSIMILATION OF CARBON DIOXIDE

In agreement with the fundamentals of photochemistry, it is found that only absorbed rays affect the assimilation of CO_2 . Timiriazeff² was the first to prove, by exact experiments carried out in the prismatic spectrum, that photosynthesis of leaves is very intensive at the absorption band of chlorophyll between B and C and that in the infra-red rays, where chlorophyll absorbs no light, not a trace of CO_2 is assimilated. This was the first proof of the rôle of chlorophyll as a sensitizer and of the subordination of photosynthesis to the principles of photochemistry. It was previously believed that the maximum of photosynthesis took place in the yellow rays which are weakly absorbed by chlorophyll. According to Timiriazeff, the quantitative relation between the absorption of different rays and the rate of photosynthesis indicates that the vigorous assimilation of CO_2 in the red rays gradually becomes less in the direction of the green part of the spectrum; the minimum of photosynthesis lies in the green rays (that is conceivable because the green rays are absorbed the least of all the other intermediate rays in the visible spectrum); then there is another increase in photosynthesis in the blue-violet rays. Thus if the assimilation of CO_2 by leaves is shown graphically on the spectrum, there are two rising curves which correspond to the first and the second maxima of photosynthesis in the red and the blue rays. At first thought, it may be surprising that there is not a whole series of high points, one for each of the absorption bands of chlorophyll; simple considerations show that this is not possible. The several absorption bands of chlorophyll appear only with low concentrations of the pigment, which are much weaker than that in living leaves. A concentration of chlorophyll equal to that normally found in leaves shows only two absorption bands in the spectrum—the one in the less bent rays stretching from B to E, and the other in the blue-violet rays.³ The change of the absorption spectrum of chlorophyllin with increase in its concentration is shown in Fig. 8 (page 74). It is evident from this figure that increasing the concentration

¹ Kostytschew, K. Bazyrina und W. Tschesnokow. *Planta*. 5: 696. 1928.

² Timiriazeff, K. The assimilation of light by plants. 1877 (Russian); *Idem*. *Ann. chim. et physique* 5: 12. 1877; cf. also Reinke, J. *Bot. Zeit.* 42: 1. 1884; Dangeard, P. A. *Compt. rend.* 152: 277 and 967. 1911; Desroche, P. *Ibid.* 153: 1014. 1911.

³ Timiriazeff. *Proc. Roy Soc. London (B)* 72: 449: 1903.

of the pigment widens the absorption band between B and C very rapidly toward to the right as far as the line E. In the blue-violet rays there is no like widening of the absorption toward the left; the several absorption bands run together but without including the green rays. Timiriazeff points out that the chloroplasts appear to be coal-black when viewed in the less refrangible rays of light and colored when seen in the green rays. When viewed in the microscope, the chloroplasts do not appear to be much colored but it must be borne in mind that their bulk color is weakened by the high magnification.

It must be emphasized that the extremely rapid widening of the most important absorption band of chlorophyll *toward the right* has not been properly valued by most investigators. This widening is the reason that the other absorption bands in the less refrangible part of the spectrum do not exist in living chloroplasts and can have no physiological effect.

The foregoing explanation shows why there can be only two assimilation maxima in the spectrum. The question now arises as to which half of the spectrum is the more important for photosynthesis. On this point, Timiriazeff performed experiments in the solar spectrum¹ and obtained the following results. If the rate of photosynthesis in the red-yellow part of the spectrum equals 100, the rate in the blue-violet end equals 54. Reinke² found the second maximum of the photochemical effect to come in the strongly refrangible part of the spectrum but since he used the method of counting gas bubbles the result is due to sources of error in his experiments. The later studies by A. v. Richter³ proved the existence of the high point in the blue-violet region and at the same time showed that the photochemical work done is greater in the red rays, as Timiriazeff also claimed. The work of Kniep and Minder⁴ confirmed Richter's results.

These pieces of work proved that the photochemical work of leaves is done at the expense of the energy of both the red and the blue-violet rays of the solar spectrum, and that in bright sunlight the red rays are the more important.

It is another question whether the ratio of efficiency, *i.e.*, the value of $\frac{U}{E}_{100}$ is constant for all portions of the spectrum; in other words, whether the same amount of photochemical work is performed per unit of absorbed energy for all wave lengths. This question is also of interest for the quantum theory (see p. 68). Both Timiriazeff and Richter touched on this question but since they both used an indirect determination of the absorbed solar energy, based on photometric measurements and theoretical calculations, their results must be considered to be indeterminate. Kniep and Minder's determination of the photochemical yield was not very exact. Timiriazeff reported that the value of $\frac{U}{E}_{100}$ was greater in the red rays than in the blue but according to Richter and Kniep and Minder the efficiency is the same in all parts of the spectrum.

¹ Timiriazeff. The photochemical effect of the extreme regions of the visible spectrum. 1893 (Russian); *Idem*. Proc. Roy. Soc. London (B) 72: 424. 1903.

² Reinke, J. *loc. cit.*

³ Richter, A. v. Rev. gén. bot. 14: 151. 1902.

⁴ Kniep und Minder. Zeitschr. f. Bot. 1: 609. 1909.

The latter results contradict the modern quantum theory. This question has now been settled by the work of O. Warburg and Negelein to be considered later.

Under natural conditions, the energy assimilated by the plant is taken from different parts of the spectrum according to the ecological and meteorological conditions. In direct sunlight the red rays are more important but in diffuse light, reflected from the blue sky, the more strongly refrangible rays are contained in greater amounts and are physiologically more significant.¹ Still greater variations from the normal composition of the sunlight are found deep under water. Since the less refrangible rays are strongly absorbed by water, the deep-water algae cannot use them at all. In this connection Engelmann² developed the interesting theory of the complementary pigments of red algae and the blue-green algae. In addition to chlorophyll, the red algae contain

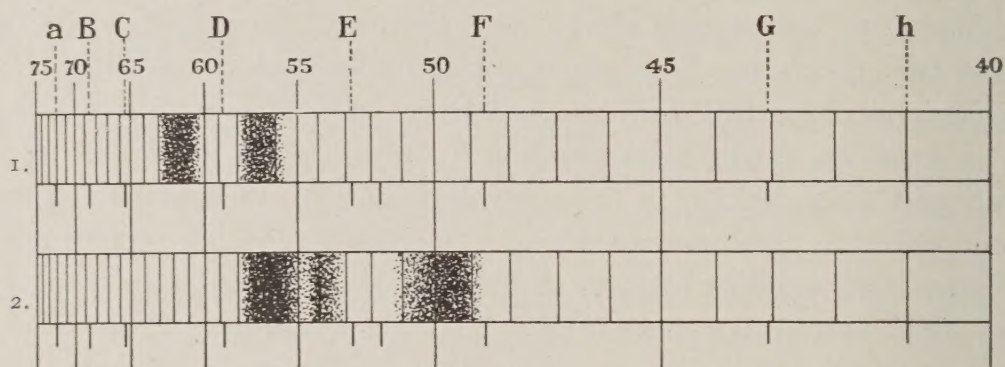


FIG. 16.—(1) Absorption spectrum of phycocyanin. (2) Absorption spectrum of phycoerythrin.

a red pigment—phycoerythrin—which has a strong orange fluorescence and is often accompanied by blue or violet-blue phycocyanin which has a red fluorescence.³ These two pigments are also found in different proportions in the *Cyanophyceae*.⁴ They have not been studied enough from the chemical standpoint. Results already cited⁵ show that phycocyanin and phycoerythrin are much alike, are united with proteins in living cells and are extracted from algae in this form by dilute solutions of salts and alkali. They can be salted out from aqueous solution and they coagulate with heat. In addition to these complementary pigments, it is also easy to demonstrate the presence of both chlorophyll and the ordinary yellow pigments in the red algae and the *Cyanophyceae*.⁶ The spectrum of phycocyanin shows a definite absorption of the orange rays between C and D (Fig. 16). Phycoerythrin also absorbs the orange rays and, in addition, the green and blue between E and F. The strong fluorescence of both pigments indicates their sensitivity to light. Their rapid bleaching in light is also remarkable.

¹ Stahl. *Biologie des Chlorophylls*. 1909; *Ursprung*. *Ber. bot. Ges.* 36: III. 1918.

² Engelmann. *Bot. Zeit.* 39: 442. 1881; 40: 419 and 663. 1882; 41: I. 1883; 42: 81. 1884.

³ Kylin. *Zeitschr. f. physiol. Chem.* 69: 169. 1910; 74: 105. 1911; 76: 369. 1912.

⁴ Teodoresco, E. C. *Rev. gén. bot.* 32: 145. 1920; cf. also Boresch, K. *Ber. bot. Ges.* 39: 93. 1921; Kylin. *loc. cit.*

⁵ Kylin. *loc. cit.*

⁶ Liebolt, F. *Zeitschr. f. Bot.* 5: 65. 1913; and others.

On the basis of his experiments with a spectroscope, Danilov¹ concludes that both these pigments assume different colors in connection with chromatic adaptation (see below). According to Danilov, the blue pigment of the red algae is not identical with phycocyanin and the red pigment of the *Cyanophyceae* is not phycoerythrin.

Engelmann (*loc. cit.*) claims that phycoerythrin and phycocyanin are complementary and photochemically active pigments of algae living in deep marine waters. These algae do not receive the red rays that are absorbed by the water but the photochemical utilization of the strongly refrangible rays by means of the complementary pigments serves them as a substitute. In this connection, Engelmann proposed the theory of "complementary chromatic adaptation" which states that many algae living in colored light have complementary colors which do not appear under normal living conditions in white light.² This chromatic adaptation occurs only under certain circumstances which are not always easily observed.³

Engelmann has not supported his theory of the photochemical activity of the complementary pigments with sufficient experimental evidence. In order to prove that the assimilation maximum for red algae is shoved toward the right of the spectrum, he used his bacterial method which is really useless for such delicate quantitative measurements. It should be taken into consideration that the intensity as well as the color of the light is significant for algae living at various depths. Algae growing under weak illumination assimilate CO₂ in all regions of the spectrum better in weak light than in strong light and *vice versa*. It is evident that a significant error may be involved in experiments on the influence of colored light in photosynthesis if this condition is not given some attention. For quantitative measurements of the photochemical output, it is essential to use the intensity of light to which the algae used are adapted.³

However, Engelmann's theory was later confirmed by exact quantitative experiments.⁴ The importance of the complementary pigments may be seen from the following table taken from Richter's paper.

If the rate of photosynthesis in direct sunlight is taken as 100, the rate of photosynthesis under red and blue light filters is expressed by the following numbers:

¹ Danilov. Mitt. d. bot. Gart. in Petersburg. 16: 357. 1916; 21: 2. 1922.

² Engelmann. Pflügers Arch. f. ges. Physiol., Suppl. Vol. 33. 1902; Gaidukov. Abh. d. Preuss. Akad. Wiss. 1902; Idem. Hedwigia. 43: 96. 1904; Idem. Ber. bot. Ges. 21: 484 and 517. 1903; 22: 23. 1904; 24: 1. 1906; Idem. Scripta Botanica. Vol. 22. 1903; Idem. Ber. bot. Ges. 41: 357. 1923; [see also Susski. Beitr. Biol. Pflanzen 17: 45-50. 1929 who worked with *Oscillatoria Engelmanniana*].

³ Boreesch. Ber. bot. Ges. 37: 25. 1919; Idem. Arch. f. Prot. 44: 1. 1921; Harder, R. Ber. bot. Ges. 40: 26. 1922; Idem. Zeitschr. f. Bot. 15: 305. 1923.

⁴ Richter, A. v. Mitt. d. Russ. Akad. Wiss. for year 1914. P. 935 (Russian). The first work of this investigator (Ber. d. bot. Ges. 30: 280. 1912) gave uncertain results which were erroneously interpreted as contradictory to Engelmann's theory. On this point see Harder, R. *loc. cit.* and Wurmser. Compt. rend. 170: 610. 1920; 171: 820 and 1231. 1920; Idem. Recherches sur l'assim. chlorophyllienne. 1921.

Alga	Red light	Blue light
<i>Ulva</i> (green alga).....	35	23
<i>Laurentia</i> (red alga).....	7	27
<i>Lithothamnion</i> (red alga).....	11	45
<i>Ulva</i> (green alga).....	12	6.2
<i>Chondrus</i> (red alga).....	7	8.6
<i>Chondrus</i> , green after escape of its red pigment.....	6	2.6

On the contrary, the brown algae are like the green algae, according to Richter, whose exact results show that their yellow and orange pigments take no part in photosynthesis.

13. THE ENERGY OUTPUT OF LEAVES UNDER VARIOUS CONDITIONS

To this section belong the technically very difficult but important investigations to determine how the solar energy is converted into chemical work in the leaf. Direct determinations of the heat of combustion of leaves before and after exposure to light gave the following results: for each gram of carbon dioxide assimilated, the plant gains 220 to 3600 calories. In Krascheninnikow's experiments,¹ 1 cm.² of leaf area assimilated 5.626 grams of CO₂ and the increase in the heat of combustion was 15,350 calories. This number is somewhat larger than the value calculated from the heat of combustion of the sugar formed in the process. For the latter, 1 gram of CO₂ should give 2552 calories (1 mol of CO₂ gives 112,300 calories) and 5.626 g. of CO₂ corresponds to 14,350 calories. From this it is clear that in addition to the sugar made in photosynthesis, there is a formation of other substances with a large heat of combustion. These are probably proteins.² Still the figures obtained from such experiments come so close to those required for the synthesis of sugar that the sugars apparently comprise most of the products of assimilation.

In his experiments with leaves of *Polygonum sachalinense* and *Acer platanoides*, Puriewitsch² produced even more distinct proof that the heat of combustion of the products of assimilation exceeds that of the carbohydrates. He left the control leaves for 24 hours in darkness while the experimental leaves were exposed to direct sunlight for 2–5 hours. Both the control and experimental leaves were then used for measurements of the heat of combustion. That of the control leaves was only a little greater than the heat of combustion of the cellulose; for *Acer* it was 4300 calories and for *Polygonum* 4423–4425 calories per gram of dry weight. The leaves exposed to light showed a greater value *per unit of dry weight*. This shows that the average heat of combustion of the products formed by photosynthesis in the leaves is greater than that of carbohydrates. Puriewitsch [*loc. cit.*] also measured the energy absorbed by the leaf

¹ Krascheninnikow, Th. The storage of solar energy in the plant. 1901 (Russian); cf. also Körösy, K. v. Zeitschr. f. physiol. Chem. 86: 368. 1913.

² This involves a change in the meaning of photosynthesis as generally accepted. Other facts bearing on the validity of the new and broader meaning of the term will appear on subsequent pages of this book. —Ed.

² Puriewitsch, K. Schriften d. Naturforscherges zu Kiew. 23: 37. 1913; Idem. Jahrb. wiss. Bot. 53: 210. 1913.

in the presence and in the absence of carbonic acid. These painstaking and difficult investigations were performed with the aid of two bolometers. The quantity of radiant energy absorbed was calculated from the difference between the amounts of energy falling on the leaf and passing through it. In the cases in which photochemical work was done, it was found that a surplus of solar energy was always stored in the leaf. The primitive measurements of Detlefsen¹ had already given the same results. The recent studies by Ursprung,² in which a Rubens thermopile was the measuring instrument, gave contradictory results; the leaves absorbed the same quantities of energy whether CO₂ was present or not. This controversy is still unsettled and it is sufficient to point out that the excess of energy in red rays used for the performance of photochemical work in Puriewitsch's experiments amounted to 11.7% of the total energy absorbed. This number is definitely greater than the experimental error. On the other hand, there was no surplus if the light was first passed through an alcoholic solution of chlorophyll. In spite of the presence of CO₂, photosynthesis was prevented in this case because the photochemically active rays were caught in the solution of chlorophyll and could not reach the surface of the leaf.

Great interest attaches the measurement of the fraction of absorbed energy that is used in photosynthesis, *i.e.*, the determination of $\frac{U}{E}100$ (p. 68). The indirect calculations³ will be mentioned only incidently because they have only historical interest. The first direct measurement was made by Brown and Escombe.⁴ They used a radiometer (a form of differential thermometer) and tried to measure the radiant energy which came to the leaf, that which was absorbed by the leaf and that used in photochemical work. The absorbed energy was calculated as the difference between that coming to the leaf and that passing through it. It was found that different plants absorb the following percentages of radiant energy:

<i>Helianthus annuus</i>	68.6%
<i>Lappa major</i>	72.8%
<i>Senecio grandifolius</i>	77.4%

However, these figures are not very exact because the light is so dispersed and reflected by its passage through the leaf.^r

¹ Detlefsen. Arb. bot. Inst. zu Würzburg. 3: 534. 1888; also Müller, N. J. C. Bot. Untersuch. Vol. 1. 1877.

² Ursprung. Ber. bot. Ges. 36: III. 1918.

³ Maquenne, L. Ann. Agronom. Vol. 6. 1880; Mayer. Amer. Jour. Sci. Vol. 45. 1893; Ursprung. Bibliotheca Botanica, pt. 60. 1903; Timiriazeff. Proc. Roy. Soc. London (B) 72: 449. 1903.

⁴ Brown and Escombe. *Ibid.* 76: 69. 1905; Brown and Wilson. *Ibid.* 76: 122. 1905.

^r Not all of the absorbed energy is used in photosynthesis for some of it would be used for such processes as transpiration. An interesting set of calculations have been made by Transeau (Ohio Jour. Sci. 26: 1-10. 1926) who based his figures on an acre of corn, for which he considered all the factors at work and concluded that at least the corn plant is very efficient in that it utilizes 46% of the energy which falls on the acre of plants. Some new data on the amount of light reflected from green plants are given by Shull, C. A. Science. 67: 107-108. 1928. His measurements were made with a spectrophotometer and he found that ordinary green leaves reflect 10 to 12% while many leaves reflect twice or three times as much.—Ed.

Brown and Escombe did not measure the photochemical output directly, but calculated it from the carbon dioxide assimilated—assuming that only sugars are the direct product of photosynthesis. It was shown above that this assumption is not quite accurate. These writers did not determine the value of $\frac{U}{E}_{100}$ and they calculated only the ratio between the energy coming to the leaf and the chemical work done. The latter was found to be about 1-4% of the energy falling on the leaf. Light absorption by leaves is a selective process and the rays passing through one leaf are absorbed only slightly by another leaf.

Puriewitsch (*loc. cit.*) used a more accurate instrument—a bolometer—in his experiments. He measured only the energy coming to the leaves. He determined the chemical output by direct measurements of the difference in the heats of combustion of leaves before and after exposure to light. It was found that the chemical output was 0.6-7.7% of the energy received. Both of Puriewitsch's methods of measurement were very accurate but of course the value of $\frac{U}{E}_{100}$ could not be calculated from his data because the fraction of energy absorbed was unknown.

It would be entirely premature to conclude from these facts that photochemical action in leaves is weak and that the leaf itself is imperfect as a photochemical apparatus. It is to be noted particularly that all of the experiments of Brown and Escombe and of Puriewitsch were performed in direct sunlight. It must be borne in mind that the production of organic substances may be limited by a single one of the various physiological facts upon which the process depends. The small fraction of radiant energy used for chemical work only shows that there is a great excess of energy at the disposal of the plant when it is illuminated by direct sunlight. Other experiments of Puriewitsch actually indicate that the fraction of energy turned to chemical use always increases as the amount of energy radiating on the plant is decreased. The change in the value of $\frac{U}{E}$ with different intensities of light may be represented by an asymptotic curve (*cf.* Fig. 5). Thus the true ratio of efficiency of photosynthesis can be measured only with weak illumination.

The latest investigations of O. Warburg and Negelein¹ as well as those of Wurmser² are especially interesting because they are trying to evaluate $\frac{U}{E}_{100}$ with low intensities of light and in monochromatic light.³ The work of Warburg and Negelein deserves particular attention in so far as they have solved the difficult problem of measuring the energy absorbed. In their experiments they used the single-celled alga *Chlorella vulgaris*. They used a suspension of the alga of such a concentration that the transmitted light could be practically

¹ Warburg, O. und Negelein. *Zeitschr. f. Elektrochem.* 102: 235. 1922; 106: 191. 1923.

² Wurmser, R. *Recherches sur l'assimilation chlorophyllienne.* 1921; [*Nature.* 124: 912-913. 1929].

³ Briggs (Proc. Roy. Soc. London (B) 105: 1-35. 1929) gives a discussion of this problem and some new data from his experiments. He questions the conclusions of Warburg and Negelein but it is true that he has used high intensities of light.—*Ed.*

disregarded. Thus the energy absorbed was equal to the energy received and this was measured with a bolometer.

The experiments were performed in monochromatic light of the lowest possible intensity. The period of illumination and that of darkness was always 10 minutes. Thus the rate of respiration was kept practically constant and the error in the determination of respiration was reduced to a minimum. The numerous measurements in various parts of the spectrum gave the following average results:

Wave Lengths of Rays	Value of $\frac{U}{E}_{100}$
Red $\lambda = 610-690\mu\mu$	59.0
Yellow $\lambda = 578\mu\mu$	53.5
Green $\lambda = 546\mu\mu$	44.4
Blue $\lambda = 436\mu\mu$	33.8

Thus it appeared that with weak illumination the photochemical output may exceed 50% of the energy absorbed. This value of $\frac{U}{E}_{100}$ is obtained in no known photochemical reaction, as indicated by the data given on page 68. It must also be borne in mind that the figures obtained by Warburg and Negelein are certainly too small because they measured the chemical work performed not by direct determination of the increase in heat of combustion of the experimental material but indirectly—by computations based on the rate of assimilation of CO_2 and on the hypothesis that the photochemical reaction is expressed by the equation $6\text{CO}_2 + 6\text{H}_2\text{O} = \text{C}_6\text{H}_{12}\text{O}_6 + 6\text{O}_2 - 674,000$ calories. It has already been shown above that the increase in heat of combustion in such experiments is always found to be greater than the value calculated according to this equation. Hence, the figures obtained by Warburg and Negelein are probably about 10% too small. It would be desirable to determine the increase in heat of combustion for *Chlorella* and to recalculate the value of $\frac{U}{E}_{100}$ on the basis of the results obtained.

It is worth noticing that the value of $\frac{U}{E}_{100}$ in the experiments of Warburg and Negelein gradually becomes smaller as the wave length decreases—in the direction red to blue. Since these experiments are the first in which the absorbed energy was measured exactly, it is to be concluded that the results obtained by Richter and by Kniep and Minder, according to which all the zones of the spectrum are equally chemically active, are to be regarded as superseded (*überholt*). The results of Warburg and Negelein correspond to Einstein's photochemical law of equivalence (*cf.* p. 68) and the equation

$$\frac{\varphi_1}{\varphi_2} = \frac{\lambda_1}{\lambda_2}$$

holds for the yellow as well as for the red rays. The equation does not hold for the blue rays because

$$\frac{\varphi_{578}}{\varphi_{436}} = 1.55 \text{ while } \frac{578\mu\mu}{436\mu\mu} = 1.32.$$

On the contrary, if the energy absorbed by the photochemically inactive yellow pigments is subtracted, the yield in the blue is too great in the sense of the quantum relationships.

Wurmser (*loc. cit.*) found that the yields in the green, blue-violet and red rays stand in the following proportion:

$$4:2.35:1.$$

These numbers seem to contradict the results of Warburg and Negelein but they really do not describe values of $\frac{U}{E}_{100}$ because Wurmser measured the total energy received but not the energy absorbed.

From their experimental data, Warburg and Negelein calculate that the decomposition of one molecule of carbonic acid requires about 5 quanta in the blue and about 4 quanta in the red and yellow rays:

Wave Length	Quanta Used
660 $\mu\mu$	4.1
578 $\mu\mu$	3.8
436 $\mu\mu$	4.7

It is therefore evident that there is a gain in energy by means of fluorescence. The fluorescent light of chlorophyll is red and gives two bands in the spectrum—from 630 to 650 and from 660 to 690 $\mu\mu$. These rays are strongly absorbed and have the highest yield. Thus, according to Warburg and Negelein, a change in the fluorescent light from yellow, green and blue must increase the value of $\frac{U}{E}_{100}$.

From all these facts it may be concluded that the green cells of plants are excellent photochemical apparatus. The recent investigations have proved beyond a doubt the significance of plants as a factor in the utilization of solar energy, since all the principles of photochemistry are found to hold for the assimilation of CO₂ in the light.

14. CHLOROPLASTS AS APPARATUS FOR PHOTOSYNTHESIS

Hitherto the leaf has always been regarded as the apparatus for photosynthesis. However, according to the results of recent work,^t the conception of the apparatus for photosynthesis is somewhat altered because the work of photosynthesis is localized exclusively in the chloroplasts while the colorless parts of the cell take no part in this work. The following facts favor this

^t Some important work not mentioned elsewhere in this chapter is that of Lubimenko (*Rev. gén. bot.* 38: 307-328, 381-400. 1926; 39: 1-115. 1927; 40: 1-59. 1928) who has experimented with chloroplasts and chlorophyll along new lines, including the isolation of chloroplasts without loss of the green color. For a critical review of this work see Priestley, J. H. *New Phyt.* 28: 197-217. 1929.—*Ed.*

idea. If two spots of light are reflected from two mirrors onto a cell of *Spirogyra* in such a way that one patch of light falls on the chloroplast and the other on the uncolored protoplasm, it can be easily shown by the use of the bacterial method that oxygen is liberated only from the lighted part of the chloroplast and not from the illuminated cytoplasm.¹ Moreover, it is possible to remove single chloroplasts from cells and to prove, using the same bacterial method, that oxygen continues to be given off for some time in the isolated condition.²

Another proof of the independence of chloroplasts in the process of photosynthesis may be deduced from the following condition. Protoplasm reacts in a certain way to chemical poisons and to mechanical stimuli (wounding) but all the processes which go on in the protoplasm are first excited by the stimulus. Then comes a period in which all the processes are retarded. If the poisoning or injury is very severe, the retardation of the protoplasmic activity is so great that death results.

This sequence of processes in irritant phenomena is just as specific and accurate a test of the activity of living protoplasm as any qualitative test in general chemistry. The most important transformations in living cells, such as respiration, growth, protoplasmic motion, etc., in fact all the vital processes, are accelerated at first by chemical or mechanical stimuli.³ This is not true of photosynthesis. Toxic substances only retard the process and the preliminary phase of acceleration is entirely lacking.³ Mechanical stimuli also fail to increase the rate of photosynthesis.⁴

The facts clearly favor the assumption that photosynthesis takes place only in chloroplasts and the cytoplasm does not share in the photochemical work. Chloroplasts consist of the pigments and the colorless stroma in which the chlorophyll is present in colloidal solution (p. 73).

On the basis of recent studies, it is possible to suppose that chloroplasts are not solid bodies but liquid drops of a colloidal solution which does not mix with the protoplasm.⁵ In this way the chloroplasts are specific media for chemical reactions and their substance is not the same as protoplasm. In fact we know that some condensation processes, such as the formation of starch, do not take place directly in the protoplasm but only in the plastids. If the plastids should prove to be liquids it might help the investigation of the mechanism of photosynthesis. Other views of the structure of plastids can be found in textbooks of plant anatomy.

Is the chlorophyll alone or is also the colorless stroma of the chloroplast active in the photochemical process? Different views have been expressed with respect to the chlorophyll. Some workers consider this pigment as nothing

¹ Engelm. Pflügers Arch. f. ges. Physiol. 57: 375. 1894.

² Idem. Bot. Zeit. 39: 446. 1881; Ewart. Jour. Lin. Soc. Vol. 31. 1896.

³ It is doubtful if this point could be proved to the satisfaction of a majority of plant physiologists. The scientific journals contain numerous reports of experiments in which only a retardation of a process can be observed.—Ed.

⁴ Irving, A. Ann. Bot. 25: 1077. 1911; Ewart. Jour. Lin. Soc. 31: 408. 1896; Treboux. Flora. 92: 49. 1903; Jakobi. Flora. 88: 323. 1899; Schröder. Flora. 99: 156. 1909; Warburg. Biochem. Zeitschr. 100: 230. 1919; Körösy, K. v. Zeitschr. f. physiol. Chem. 93: 145. 1914; Schmucker, T. Biochem. Zeitschr. 195: 149. 1928.

⁵ Kostytschew. Ber. bot. Ges. 39: 328. 1921.

⁶ Ponomarew, A. Arb. d. Naturforscherges in Kazan, no. 296. 1914; Idem. Ber. bot. Ges. 32: 483. 1914.

more than a sensitizer.¹ Others believe, without misunderstanding this rôle, that chlorophyll also takes part in the chemical reactions of photosynthesis.² A third group go still farther and claim that the whole process of photosynthesis is localized in the green pigment alone.³

The importance of chlorophyll as a sensitizer is to be regarded as definitely proved. It has been shown that the reduction of silver salts in the presence of chlorophyll takes place in the red rays between B and C, although the silver salts can be reduced photochemically only in the strongly refrangible rays.⁴ It is evident that chlorophyll activates the rays which it absorbs.⁵ Still the idea of the sensitizer theory is not entirely clear; perhaps it is aided by the latest results which show that fluorescent substances exert a photodynamic effect. This effect is said to be produced by a stream of electrons which are developed by the fluorescence.⁶ At present all experiments to demonstrate a connection between the fluorescence of chlorophyll and its action as a sensitizer⁷ have unfortunately led to negative results.⁸

If the importance of chlorophyll as a sensitizer is considered proved, it is otherwise with efforts to ascribe to chlorophyll the whole chemical work of photosynthesis. The simplest and clearest proof of this idea would be to demonstrate photosynthesis in the presence of chlorophyll removed from the leaf and chloroplasts. Such experiments have often been attempted and success has been reported several times. At first it was considered sufficient to prove the liberation of oxygen from killed plants in the light, using sensitive qualitative methods.⁹ Then the attempt was made to reduce CO₂ and form formaldehyde by the use of isolated chlorophyll preparations. Later chlorophyll was used in solid form or in colloidal solutions.¹⁰ Careful repetition of all these experiments gave negative results, unfortunately.¹¹

¹ Timiriazeff. *Compt. rend.* 96: 375. 1883; 102: 686. 1886; 109: 379. 1889; *Idem.* *Proc. Roy. Soc. London (B)* 72: 421. 1903; Mazé. *Compt. rend.* 160: 739. 1915; Czapek. *Biochem. d. Pflanzen* 1: 613. 1913; and others.

² Willstätter und Stoll. *Untersuchungen über die Assimilation der Kohlensäure* 1918; Fischer, E. *Jour. Chem. Soc. (London)* 91: 1749. 1907; and others. [Emerson, R. *J. Gen. Physiol.* 12: 623-639. 1929.]

³ Usher and Priestley. *Proc. Roy. Soc. London (B)* 84: 101. 1911; Moore, B. and T. A. Webster. *Ibid.* 87: 163. 1913; Ewart. *Ibid.* 89: 1. 1915; and others.

⁴ Becquerel. *Compt. rend.* 79: 185. 1870; Timiriazeff. *Ibid.* 100: 851. 1885. Confirmations: Liesegang. *Chem. Centralbl.* 1: 636. 1894; Neuhauss. *Photogr. Rundschau.* 16: 1. 1902.

⁵ The supposed existence of the so-called "green bacteria" with inactive chlorophyll is contradicted by the still unpublished work of Perfilieff.

⁶ Tappeiner, H. v. *Ergeb. d. Physiol.* 8: 698. 1909; Hausmann und Portheim. *Biochem. Zeitschr.* 21: 10. 1909; 30: 276. 1911; *Idem.* *Ber. d. bot. Ges.* 26a: 452. 1908; *Idem.* *Jahrb. wiss. Bot.* 46: 599. 1909; Stark. *Physikal. Zeitschr.* 8: 81 and 248. 1907; Vol. 9. 1908.

⁷ Tswett, M. *Zeitschr. f. physikal. Chem.* 76: 413. 1911.

⁸ Richter, A. v. *Mitt. Russ. Akad. Wiss. for* 1914. P. 857. (Russian)

⁹ Friedel. *Compt. rend.* 132: 1138. 1901; Macchiati. *Rev. gén. bot.* 15: 20. 1903; Molisch. *Bot. Zeit.* 62: 1. 1904.

¹⁰ Usher and Priestley. *Proc. Roy. Soc. London (B)* 77: 369. 1906; 78: 318. 1906; 84: 101. 1911; Schryver, S. B. *Ibid.* 82: 226. 1910; Chodat, R. et K. Schweizer. *Arch. Sci. Phys. et Nat. Genève* (4) 39: 334. 1915.

¹¹ Herzog, R. O. *Zeitschr. f. physiol. Chem.* 35: 459. 1902; Ewart, A. J. *Proc. Roy. Soc. London (B)* 80: 30. 1908; Euler, H. v. *Zeitschr. f. physiol. Chem.* 59: 122. 1909. On this point cf. especially Willstätter und Stoll. *Untersuchungen über Assimilation der Kohlensäure*, p. 371. 1918; as well as Jörgensen, J. and F. Kidd. *Proc. Roy. Soc. London (B)* 89: 342. 1917.

Different investigations have been devoted to the behavior of chlorophyll in living leaves. Many workers think that chlorophyll must be continually decomposed and reformed in the chloroplasts.¹ An apparent confirmation of this hypothesis is found in the instability of chlorophyll dissolved in organic solvents. Those workers who not only ascribe to chlorophyll a rôle of sensitizer but also emphasize the chemical action of chlorophyll in the reaction involved in photosynthesis, often claim that chlorophyll is the first product of photosynthesis. Chlorophyll is then said to break up so that on one hand the normal products of photosynthesis appear and accumulate, and on the other hand oxygen escapes and the decomposed chlorophyll is regenerated by the addition of CO₂. Such a picture of an autocatalytic process is said to be acceptable because the formation of chlorophyll is actually a photochemical reaction and chlorophyll is formed most rapidly by the action of rays which are also particularly active in the photochemical assimilation of CO₂.^v Hence, it is said to be proper to suppose that this is more than a coincidence.

Some investigators have tried to prove that formaldehyde is actually formed in the photochemical cleavage of chlorophyll,² since it is usually regarded as the first product of the reduction of carbonic acid in the light. In this connection there have been various theories of the chemical changes of chlorophyll in the leaf. However, there has been no suitable proof of the formation of formaldehyde [from chlorophyll] and the theories based thereon are more or less improbable. Since quantitative determinations of the leaf pigments have now become feasible, it can be proved whether the amount of chlorophyll in a leaf varies or not under different conditions. Numerous analyses have shown that both the total amount of green pigment and the ratio of the components *a* and *b* remain constant at different hours of the day.³ This would be hard to explain from the standpoint of these theories. The ratio of *a* to *b* does not even change under the action of poisons. An especially convincing proof of the stability of chlorophyll in the leaf is furnished by the determination of its amount when the rate of photosynthesis is artificially increased by raising the temperature, illumination and CO₂-content.⁴ Under these conditions the leaves function about ten times as fast as under natural conditions, yet the quantities of the pigments undergo no change even in a long experiment. For example, in such an experiment 10 g. of leaves of the laurel cherry worked continually for 22 hours at such a rate that they assimilated 1.12 g. of CO₂. The chlorophyll content was 9.4 mg. at the start and 9.5 mg. after the experi-

¹ Timiriazeff. The spectrum analysis of chlorophyll. 1872 (Russian); Wiesner, J. Sitzungsber. d. Akad. Wien, Math-nat. Kl. 69: 159. 1874; Idem. Bot. Zeit. 32: 116. 1874; Stokes, G. G. On light. P. 285. 1892; Pfeffer, W. Pflanzenphysiologie. Vol. I, p. 318. 1897; Czapek, F. Ber. bot. Ges. 20: 44. 1902 (A summary); Idem. Biochem. d. Pflanzen. 1: 615. 1913.

^v New support for an autocatalytic reaction in photosynthesis has been given by the work of Emerson, R. (J. Gen. Physiol. 12: 623-639. 1929) who obtained his evidence from a mathematical analysis of curves plotted from very accurate measurements of the gas exchanges in photosynthesis at different temperatures.—Ed.

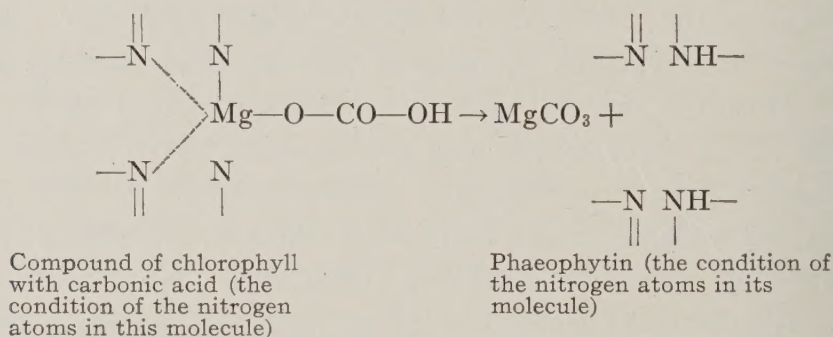
² Warner, C. H. Proc. Roy. Soc. London (B) 87: 378. 1914; Wager, H. Ibid. 87: 386. 1914; Ewart, A. J. Ibid. 89: 1. 1915.

³ Willstätter and Stoll. Untersuchungen über Chlorophyll, p. 109. 1913.

⁴ Idem. Untersuchungen über Assimilation der Kohlensäure, p. 39. 1918. These results have been contradicted recently by Wlodek, J. Bull. Acad. Polon. (B), pp. 19 and 143. 1921.

ment. *Pelargonium* leaves did not lose a trace of chlorophyll after working just as fast for 6 hours at 40°; before the experiment they contained 12.5 mg. and afterwards 12.8 mg. of pigment. It can hardly be supposed that chlorophyll could be formed so rapidly that there would not be a loss during the decomposition and synthesis presupposed by this theory.^w It is much more probable that chlorophyll in living cells is a stable substance under those circumstances. Of course it is uncertain as to what gives this stability to the pigments which so readily undergo change in molecular solutions outside of the chloroplasts.

Willstätter and Stoll assume that the radiant energy is transformed into chemical work in the chloroplast itself while the chlorophyll is taking part in the first reactions of photosynthesis. These workers showed that chlorophyll does fix carbon dioxide.¹ It has already been pointed out that magnesium is removed from chlorophyll as a carbonate under the action of CO₂. This takes place only in aqueous, colloidal solution and not in organic solvents. At low temperatures the CO₂ unites with the chlorophyll to form a labile substance which readily changes to phaeophytin and magnesium carbonate under the influence of an increase in temperature and other changes in the conditions. Since the chlorophyll is not bleached by forming compounds with CO₂, it is fair to assume that the complex union with magnesium is uninjured and that only one valence of magnesium is united to the carbonate.



Willstätter and Stoll think that carbonic acid is united with chlorophyll by reduction and other steps which lead to the formation of organic substances. In any case, there is a suggestion of the formation of carbonic acid-chlorophyll in that the green pigment actually plays the rôle of a substance chemically active in photosynthesis.

The numerous determinations of the ratio between the amount of chlorophyll and the chemical work done by the leaf² are of interest in two respects. In the first place, it is definitely proved that photosynthesis depends not on the chlorophyll alone but also on the other substances contained in the plastids.^x Secondly, they explain why the experiments (described on pages 100 ff.) to measure the fraction of total available energy that is used in photosynthesis, so often gave uncertain results. It could not be otherwise. The work done per unit weight of chlorophyll per hour ("assimilation number") varies through

^w On the contrary, synthesis and decomposition could be expected to take place at exactly the same rate if these changes are a part of a chemical reaction which is in dynamic equilibrium. We are finding so many such systems in physiology that we cannot assume that stability of a substance involves a static condition.—Ed.

¹ Willstätter and Stoll. Untersuchungen über Assimilation der Kohlensäure, p. 228. 1918.

² loc. cit., pp. 41-161.

³ This point was recently taken up again by Emerson, R. (J. Gen. Physiol. 12:609-622. 1929) who used Warburg's methods and pure cultures of *Chlorella vulgaris* in which he could vary the chlorophyll content at will by controlling the supply of iron. He came to the conclusion that "the maximum rate of photosynthesis is a smooth function of the chlorophyll content" and the accuracy of his methods and data leaves no room for doubt in the matter.—Ed.

a wide range. It is proportionally small for leaves rich in chlorophyll and becomes larger for leaves with little chlorophyll (leaves just turning green, autumn leaves and the like). This number is especially high for the yellow-leaved varieties of various plants. Both types of leaves perform about the same amount of work in bright light under favorable conditions. In other words, each molecule of chlorophyll in leaves poor in chlorophyll does just as much work as in leaves with plenty of chlorophyll. For example, the following figures were obtained for the elm;¹

	Chlorophyll in 10 g. of leaves, mg.	CO ₂ assimilated per dm ² per hour, mg.	Assimilation number
Green variety.....	16.2	21	6.9
Yellow variety.....	1.2	24	82
Green variety.....	13.7	30	9.9
Yellow variety.....	1.5	29	73

It is evident that these leaves in direct sunlight perform a certain amount of chemical work with about a tenth of the chlorophyll contained in them. On this basis, each molecule of chlorophyll in leaves rich in it averages a low output of energy. In spite of this, the leaf cannot increase the amount of work done because only the sensitizer (chlorophyll) is present in excess while the other factors which take an active part in photosynthesis cannot increase their activity. Hence, it is quite conceivable that, as calculation shows, a molecule of chlorophyll in normal elder leaves handles 135 molecules of CO₂ per hour and 2463 molecules of CO₂ per hour in leaves of the yellow strain of the same plant. Chlorophyll is only one of several factors active in the assimilation of CO₂. The small chemical performance in comparison with the energy received, which Brown and Escombe (p. 101) obtained in direct sunlight must be 20 to 40 times as large in the case of the yellow strains with which Willstätter and Stoll worked. Still greater variations in output are to be expected from changes in illumination. In diffuse light we find no surplus of chlorophyll in normally colored leaves and the output of energy increases rapidly as the light intensity falls off (*cf.* p. 102). An increase of 90–100% in the output of energy has also been observed for alternating dark and light conditions.²

As for the colorless “stroma” of the chloroplasts, this includes various important substances and enzymes. Its importance can be stated only hypothetically. It is extremely probable that it takes a direct part in the process of photosynthesis. An interesting fact which may be related is the activity of the leaf substance in absorbing considerable amounts of CO₂, depending on the CO₂ tension in the surrounding atmosphere.³ This fixation of CO₂ by the leaf substance is quite different from that in which the pigment takes part.

¹ *loc. cit.*

² Warburg, O. *Biochem. Zeitschr.* 100: 203. 1919.

³ Willstätter und Stoll. *Untersuchungen über Assimilation der Kohlensäure*, p. 226. 1918; Spoeher and McGee. *Science*. 59: 513. 1924.

It occurs in darkness as well as in the light and equally in leaves rich and poor in chlorophyll. Spoehr and McGee (*loc. cit.*) believe that this process is due to the formation of carbamino acids from proteins and their cleavage products. This concentration of carbonic acid near the chlorophyll seems to have the important physiological function of supplying CO_2 to the leaf when it is working in an atmosphere containing very little of it. It is very interesting that the leaf takes up CO_2 at least half as fast as concentrated potassium hydroxide.¹ At the same time, it has been shown that the epidermis and stomates are so constructed that when the stomates are open the inner parenchyma of the leaf is ventilated just as well as though there were no epidermis present. Still the rate of absorption of CO_2 is such that we could not understand it if this property of the leaf tissue to fix CO_2 loosely were not known.

Another interesting characteristic of chloroplasts is their strong reducing power. If living tissue is treated with a silver salt, the chloroplasts at once turn black.² Leucoplasts, which remain colorless, fail to reduce silver and dead chloroplasts are equally inactive. The reduction is probably brought about by some aldehyde which is contained in the chloroplast and which represents a product of the photosynthetic process.

This process goes on so rapidly in comparison with other chemical reactions in plants that it can be observed through a microscope. The results of studies of peculiar chondriosome-like structures which are formed in the chloroplasts during photosynthesis and then seem to pass into the surrounding cytoplasm in the fluid form,³ may be interpreted as phenomena which are associated with the metabolism of nutrition. It may be pointed out in this connection that the "droplets of oil" in the chloroplasts of many seed-plants and the alga *Vaucheria* have recently been called a special secretion of assimilation that has nothing to do with oil.⁴ All these inclusions form in the light and disappear in darkness.

15. THE PRODUCTS AND MATERIALS OF PHOTOSYNTHESIS

The principal organic substance synthesized by photosynthesis is carbohydrate. Proteins are also formed in relatively small quantities. Thus carbohydrates and proteins arise from inorganic substances in the light and represent the basic material for the synthesis of all other organic compounds, in animals as well as in the plants. The carbohydrates are very well suited to be the working material of vital processes. Moreover, they dissociate in solution and can thus change easily into other physiologically important substances. The proteins are also very well adapted to the formation of the other nitrogenous compounds in plant cells.

The first demonstration of the accumulation of carbohydrates in leaves in the light was made by Sachs⁵ by the following ingenious method. It had been known for some time that chloroplasts contain starch grains. If plants are left for some time in darkness, the starch gradually disappears from the

¹ Brown, H. T. and Escombe. Phil. Trans. Roy. Soc. London (B) 193: 223. 1900; Sierp, H. und K. Noack. Jahrb. wiss. Bot. 60: 459. 1921.

² Molisch, H. Anz. Akad. Wiss. Wien. Vol. 55: 1918; Idem. Ber. bot. Ges. 39: 136. 1921.

³ Lewschin, A. Experimental-cytological investigation of mature leaves of autrophic plants in relation to the question of the nature of chondriosomes. 1917 (Russian).

⁴ Meyer, A. Ber. bot. Ges. 35: 586 and 674. 1917; 36: 235. 1918.

⁵ Sachs. Bot. Zeit. 20: 365. 1862; 22: 289. 1864.

leaf. If the latter is then removed from the plant and exposed to sunlight or a strong artificial light, with a portion of the illuminated leaf surface covered with heavy, dark paper, it is easy to show that starch is formed only in the places directly exposed to the light. To do this, remove the color from the leaf with hot alcohol and then apply an alcoholic solution of iodine. Iodine imparts a blue or violet color to the starch grains.¹ The dark color appears only in the parts of the leaf which are directly exposed to light (Fig. 17). This test, which clearly proves the dependence of starch formation upon light, is called "Sachs' Iodine Test." An essential supplement to it is the demonstration of the dependence of the formation of starch upon the presence of carbon dioxide in the surrounding atmosphere. In air free from CO₂, the iodine test gives a negative result, even in very bright light.² Since the rapid formation of starch in the light can be demonstrated, very often after a few minutes,³ starch was at first considered to be the first visible product of photosynthesis. Now we know that this is incorrect. Only the surplus of the carbohydrate formed is deposited in the form of starch, which is quite appropriate. The solid grains of starch do not increase the osmotic pressure in the cell, do not retard the normal course of carbohydrate formation according to the law of mass action, and are temporarily kept from further changes. Thus starch is like a surplus income which is put in the savings bank after defraying all unavoidable expenses.

By the following ingenious experiment, Timiriazeff⁴ showed that starch is formed chiefly under the influence of the photochemically active rays. He projected a small bright spectrum on a leaf free from starch. After exposure to the spectrum, the iodine test proved that starch was most abundantly formed in the red rays between B and C and from there on the amount of starch decreased gradually to the right. Almost no starch was formed in the blue and violet rays although the second maximum of CO₂ assimilation is found here. This result, confirmed by later observations,⁵ is a new proof that starch is a secondary product and that the formation of starch cannot be used as a measure of photosynthesis. The absence of the formation of starch in the blue-violet rays, as well as the results of Warburg and Negelein (p. 103), according to which there is a theoretically lower output of energy in the blue, will be considered below.

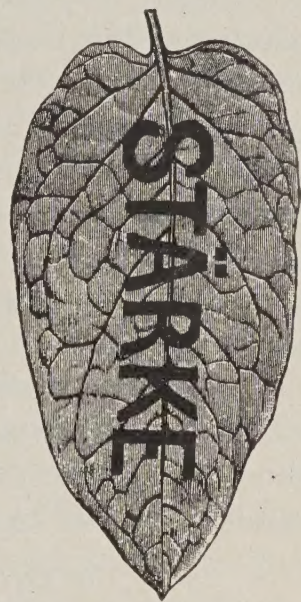


FIG. 17.—Sachs' starch test. Starch (*Stärke*) has been formed only in the illuminated parts of the leaf. After treatment with iodine, the parts covered with paper have not given the characteristic color reaction of starch.

¹ In some cases the leaf can also be treated with a solution of iodine in ether which readily penetrates the stomates. Cf. Neger. Ber. bot. Ges. 30: 93. 1912. [A new method reported by Emerson, F. W. (Science. 65: 598-599. 1927) involves the use of gasoline as the solvent of the iodine.]

² Godlewski, E. Flora, p. 378. 1873.

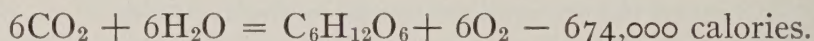
³ Faminzyn. The action of light on algae. 1886 (Russian); Kraus, G. Jahrb. wiss. Bot. 6: 511. 1869. Godlewski, E. Bot. Jahresber. for 1875, p. 788.

⁴ Timiriazeff. Compt. rend. 110: 1346. 1890.

⁵ Ursprung, A. Ber. bot. Ges. 36: 86. 1918.

Later investigations showed that soluble sugars are the first, demonstrable, nitrogen-free products of photosynthesis.¹ Cane sugar is usually quantitatively the most abundant sugar, making up 50–90% of all the sugar present,² but it certainly is a secondary product, the result of a condensation of the hexoses—glucose and fructose.³ These simple sugars may easily be interchangeable in plants so that it is difficult to prove which of them represents the direct product of photosynthesis. Aside from cane sugar, there are other sugars which appear in leaves.⁴ Plants were once classified as starch plants, sugar plants and oil plants but this classification is now discarded because it is known that in the “starch plants” the starch often constitutes but a small fraction of the total result of photosynthesis,⁵ that the supposed “oil” is to be regarded as a “secretion of assimilation” which may contain intermediate products of the assimilation of carbon and the formation of sugar.⁶ According to the present views, the difference between these three categories is not qualitative but only quantitative; by increasing the rate of photosynthesis in plants which usually form no starch, it is easy to demonstrate the formation of this reserve substance.⁷

So all the experimental evidence at our disposal shows that simple sugars are the first demonstrable, nitrogen-free products of photosynthesis.^y Since the simple sugars predominate, the main trend of the photosynthetic process can be expressed by the equation:



This equation states that plants form their organic substances at the expense of the carbon of carbon dioxide, the elements of water and the energy of light. Of the four *Organogens* (*i.e.*, elements from which the physiologically important organic compounds are put together) the plant obtains three of them from such simple inorganic substances as CO_2 and water.

It has been mentioned frequently in this chapter that atmospheric carbon dioxide is the source of carbon for green plants. This view was represented by Ingen-Housz, the founder of the knowledge of the nutrition of plants by means of light,⁸ who defended it against Hassenfratz,⁹ the originator of the “humus theory” (of which more will be said later).

¹ Brown, H. and Morris. Jour. Chem. Soc. Trans. 63: 604. 1893.

² Davis, A. and A. J. Daish. Jour. Agr. Sci. 5: 437. 1913; 6: 152. 1914; Gast, W. Zeitschr. f. physiol. Chem. 99: 1. 1917.

³ Weevers, Th. Kon. Acad. Wet., Amsterdam. 27: 46. 1923.

⁴ Kylin, H. Zeitschr. f. physiol. Chem. 101: 77. 1918; Michel-Durand, E. Rev. gén. bot. 30: 337. 1918.

⁵ Brown, H. and Morris. *loc. cit.*; Kylin. *loc. cit.*; Körösy, K. v. Zeitschr. f. physiol. Chem. 86: 368. 1913; Michel-Durand, E. *loc. cit.*

⁶ Meyer, A. *loc. cit.*

⁷ Schimper. Bot. Zeit. 43: 737. 1885; Godlewski, E. Flora. 60: 218. 1877.

⁸ It is still a debated question as to which is the primary sugar—sucrose or hexoses. A number of writers have argued that sucrose is the first. These include Brown and Morris (*loc. cit.*), Parkin (Biochem. Jour. 6: 1. 1911), Went (Jahrb. wiss. Bot. 31: 323. 1898), Perry (Compt. rend. 94: 1124. 1882) and Girard (*Ibid.* 97: 1305. 1883). Most workers seem to agree that sucrose can not be the first sugar and in addition to the points mentioned above, there are others who have produced evidence in support of the opposite view. The problem was reviewed by Priestley (New Phyt. 23: 255–265. 1924) and Spoehr (Photosynthesis. 1926. P. 216 ff.) has also stated both sides of the case.—*Ed.*

⁹ Ingen-Housz, J. See p. 66.

⁹ Hassenfratz, J. H. Ann. d. chim. 13: 178. 1792; Vol. 14. 1792.

Because these two scientists were unable to confirm their views by quantitative analyses, they waged a battle of words and could not solve the problem. Hassenfratz thought that most of the carbonic acid used by plants came to the leaves in the water current rising from the roots and he defended his position by pointing out that the CO_2 content of the air is too low to meet the needs of the plant. This objection was again raised in the 19th century, although the classical experiments of de Saussure,¹ which were more accurate than the work of his contemporaries and even of many later workers, proved an energetic assimilation of atmospheric CO_2 by green plants and demonstrated that plants could not live in air free from CO_2 . In the first half of the 19th century, the humus theory was still widely accepted and even in the year 1855 Unger² tried to show that plants are dependent on the carbonic acid of the soil. The correct view was first established by Boussingault³ who showed by means of accurate experiments with currents of air that the atmospheric CO_2 sufficed for the nutrition and normal development of plants.

One cannot wonder at the sustained doubt with respect to the importance of the atmospheric carbonic acid. The CO_2 content of the air is so small that it is only by reason of the remarkable structure of the leaf that the plant is able to manufacture enough organic substance for its existence. The numerous determinations of CO_2 in the atmosphere⁴ made during the present century show that the CO_2 content fluctuates between 0.0243% and 0.0360% and averages about 0.0294%. The older investigations gave similar results. The CO_2 content of the air over the sea is the same as that over the land⁵ but just above the surface of the earth we find 0.12–0.13% CO_2 , which must have a favorable effect on the photosynthesis of the herbaceous plants.⁶ These plants are thus actually able to utilize the carbonic acid of the soil but only as it escapes into the atmosphere.⁷ Thus we are now certain that Ingen-Housz was right in the great dispute.

The carbon dioxide reaches the internal intercellular spaces of the leaf by way of the stomates, along with the other components of the atmosphere. As a result of the wonderful system of aeration in the leaves, the cells of the assimilating tissues are bathed in the gases of the atmosphere as freely as though there were no epidermis. It has already been pointed out that the leaf absorbs atmospheric carbon dioxide at least half as fast as the absorption by the surface of a concentrated solution of potash.⁷ Brown and Escombe studied the rate of diffusion of gases through small openings and concluded that it is not directly proportional to the area of the opening as might be expected. For several

¹ Saussure, Th. de. *Recherches chim. sur la végétation*, Sect. 5. 1804.

² Unger. *Anatomie und Physiologie der Pflanzen*. 1855; cf. also Moleschott, J. *Physiologie des Stoffwechsels*, p. 58. 1854.

³ Boussingault. *Agronomie, chim. agr. et physiol.* 3: 266. 1864; 4: 267. 1868; 5: 7. 1874.

⁴ Brown, H. T. and F. Escombe. *Proc. Roy. Soc. London (B)* 76: 118. 1905; Giltay. *Ann. jard. Buitenzorg*. 15: 57. 1898; cf. especially Lundegårdh, H. *Der Kreislauf der Kohlensäure in der Natur*. 1924.

⁵ Legendre, R. *Compt. rend.* 143: 526. 1906; Schröder, J. *Chem. Zeit.* 35: 1211. 1911.

⁶ Wollny. *Forsch. Agr.-Physik.* 8: 405. 1885; Demoussy. *Compt. rend.* 138: 291. 1904.

⁷ There have been observations reported which indicate that there is no great fertilizer effect, acting directly on the lower strata of vegetation, from the CO_2 which escapes from the soil. For example, Hasse, P. and Kirchmeyer (*Zeitschr. Pflanzenernähr. Düng und Bodenk. A. Wiss. Teil.* 10: 257–298. 1928) confirmed the earlier views of Lemmermann (published in the same journal) that this type of fertilizer effect is unimportant. The data of Hasse and Kirchmeyer show an increase in CO_2 content of only 2–6% in the vicinity of leaves of rye, potatoes, etc.—Ed.

⁷ Brown, H. T. and F. Escombe. *Phil. Trans. Roy. Soc. London (B)* 193: 223. 1900; Sierp, H. und K. Noack. *Jahrb. wiss. Bot.* 60: 459. 1921.

reasons the relative rate of diffusion increases as the breadth of the opening diminishes. An opening with an area of 1 mm.² transmits less gas per unit of time than 10 small slits with a combined area of 1 mm.² If the area of the slits and the ratio of the rate of diffusion to the total opening is known, the distance between openings can be so calculated that the diffusion through the partition wall goes on at a rate which corresponds to the rate of diffusion through an opening which equals the whole area of the partitions—in other words, the rate of diffusion without any partitions. This calculation shows that the epidermis of the leaf with the stomates open is an example of the arrangement by which the relative rate of gas diffusion reaches a maximum.^a In order to picture the perfection of leaf structure it is sufficient to connect with these results of Brown and Escombe the results of other experimental studies which have shown that photosynthesis is inhibited by closed stomates because the diffusion of gas through the cuticularized walls of the epidermal cells is negligible.¹

When the stomates are open the result is conditions of diffusion which amount to a complete removal of the membrane but when they are closed the intercellular spaces of the leaf are separated from the outside atmosphere by a wall that is impermeable to gases. It must also be borne in mind that such partitions apply only to photosynthesis and not to other functions, such as transpiration. With the stomates half closed, photosynthesis continues while the transpiration is considerably retarded. In this connection it is necessary to notice that the leaf is usually a flat, dorsi-ventral organ in which the upper and lower sides do not have the same structure. Most of the stomates usually occur on the under side and the specific tissue for assimilation is the palisade parenchyma which lies just beneath the upper epidermis. On the whole the leaf is a complicated apparatus which is so adjusted to the photosynthetic function of the chloroplasts that light can act on the greatest surface possible. Hence it would be incorrect to calculate the photosynthetic yield according to the weight or volume of the leaf and not according to the surface. Thorough studies show that it is sufficient to invert the leaf and thus expose the under side to the light, in order to weaken the assimilation of CO₂ although neither the weight nor the volume of the leaf is altered.²

The water plants are nourished by the free carbonic acid in solution in the water and by the carbonic acid of carbonates. In aqueous solution the carbonic acid diffuses directly through the thin cell walls of the delicate assimilating organs of the deep-water plants.³ With reference to the bicarbonates, the idea was once held that they cannot be assimilated directly but that they

^a As the result of a mathematical study of laws of evaporation from small areas, **Jeffreys** (Phil. Mag. 35: 270–280. 1918) came to the conclusion that Brown and Escombe's estimate of 10 diameters between stomates for independent action of diffusion through them is too low.—*Ed.*

¹ **Blackman, F. F.** Proc. Roy. Soc. London, Vol. 57. 1895; **Idem.** Ann. Bot. 9: 164. 1895; **Brown, H. T. and F. Escombe.** Proc. Roy. Soc. London (B) 76: 61. 1905. Cf. also the older papers: **Boussingault.** Agronomie, chim. agr. et physiol. 6: 357. 1878; **Mangin.** Compt. rend. 105: 879. 1887; **Stahl.** Bot. Zeit. for 1894, p. 129; **Nagamatz.** Arb. d. bot. Inst. zu Wurzburg. 3: 389. 1887.

² **Boussingault.** Agronomie, chim. agr. et physiol. 4: 359. 1868; **Meissner, R.** Dissertation. Bonn. 1894.

³ **Devaux, H.** Ann. Sci. Nat. (7) 9: 35. 1890.

dissociate as the concentration of free carbonic acid is lowered and the content of free carbonic acid is thus maintained at a constant level. Under normal conditions, this level is not high because it depends on the partial pressure of the CO_2 of the air.¹ In the case of the photosynthesis of the water plants, the dissolved bicarbonate changes to carbonate as the water becomes less alkaline. Hence there has been a change in the idea that the photosynthesis of water plants is less than that of land plants because this is hardly correct. The alkalinity of water containing carbonates sometimes goes so high ($\text{pH} = 9.5 - 9.8$) that there is no doubt but that the bicarbonates are used by the plants.² In respect to the assimilation of hydrogen and oxygen, it takes place at the expense of the water as de Saussure proved.³ In one of his classical experiments the dry weight of the plant after photosynthesis had reached 531 mg. while only 217 mg. of carbon dioxide had been taken in: the other 314 mg. corresponded to the water taken in. The ratio of water to carbon was 1.447 which approximated the theoretical value of 1.5 for the formation of sugar. Unfortunately there have been no direct determinations of water assimilation in recent times. These measurements involve technical difficulties but it is so important to study the assimilation of water that this gap ought to be filled.

Naturally it is scarcely conceivable that the ratio of carbon to hydrogen and oxygen should always remain constant for photosynthesis because proteins are also formed. Direct analytical determinations have shown that in some cases the protein amounts to 30% of the products of photosynthesis.⁴ In addition to carbon, hydrogen and oxygen, it is necessary to have nitrogen, sulphur and phosphorus to make proteins. All these elements are taken in by green plants in the form of simple, inorganic compounds (Chap. V).

By means of classical experiments, which were the pioneer studies in the physiology of plant nutrition, Boussingault showed that the molecular nitrogen of the atmosphere is not assimilated by seed-plants⁵ (without the assistance of bacteria). In one famous experiment he grew three sunflower plants in sterile sand. The first plant was watered with pure water and grew on the



FIG. 18.—Sunflower plants. (1) given salt-peter, (2) given no nitrogen. (After Boussingault.)

¹ Cf. Wilmott, A. J. Proc. Roy. Soc. London (B) 92: 304. 1921.

² On this point cf. Ruttner, F. Sitzungsber. d. Akad. Wien, Math-natur. Kl. I. 130: 71. 1921; James, N. O. Proc. Roy. Soc. London (B) 130: 1. 1928; according to Schutow (Planta. 2: 132. 1926) the carbonic acid of calcium carbonate is utilized.

³ Saussure, Th. de. loc. cit.

⁴ Saposchnikoff, W. Proteins and carbohydrates as assimilation products of green leaves. 1894 (Russian); Idem. Ber. bot. Ges. 8: 233. 1890. [For an adverse criticism of this work see Spoehr. Photosynthesis. 1926. P. 220.]

⁵ Boussingault. Agronomie, chim. agr. et. physiol. 1: 1. 1860.

material stored in the seed. The second plant received a solution of mineral salts suitable for plant growth except that the nitrogenous compounds were omitted. The third plant received the complete mineral solution in which nitrogen was present as potassium nitrate. All the plants were kept under large glass bell-jars sealed with sulphuric acid which prevented the entrance of air containing ammonia or some oxidized compound of nitrogen. The water and carbonic acid for the nutrition of the plants were admitted through glass tubes so that the bell-jars were not opened throughout the experiment.

The following results were obtained 86 days after the seed was planted. The plants watered with saltpeter had developed perfectly normally. Plants which did not receive nitrogen made very little growth and were dwarfed at the end of the experiment (Fig. 18, 2). Their growth was limited by the small store of nitrogen in the seed. Expressed in figures, the results of the experiment are conclusive:

Plant grown	Increase in dry weight, mg.	Increase in nitrogen, mg.
In pure sand.....	0.28	2.3
In sand plus mineral salts.....	0.39	2.7
In sand plus mineral salts plus KNO ₃	21.11	166.7

There is no doubt but that the "increase in nitrogen" in the plants which grew without a supply of nitrogen falls within the limit of error of the experiment, since the same amount was calculated from control portions of seeds which were not germinated but analyzed directly. There are small individual variations in the nitrogen content of seeds.

Boussingault thus proved that plants of the normal type cannot use the nitrogen of the atmosphere. His experiments are also interesting because they emphasized the great importance of nitrates for the nutrition of green plants. Organic derivatives of oxidized nitrogen have never been found in plants and before Boussingault's work it was thought that ammonium salts are the best source of nitrogen for green plants. It should be noted especially that ammonia may be used directly to form amino acids, the building blocks of the protein molecule, while nitrates must first be reduced at the expense of considerable outlay of energy. The statements in the literature concerning the oxidation of NH₃ to nitric acid by seed-plants¹ have not been confirmed and it is usually thought that all the nitrates in plants is taken directly from the soil through the roots.² In some plants considerable amounts of nitrates accumulate under suitable conditions; the *Urticaceae* and *Chenopodiaceae* are characteristic plants of this type.³

Inorganic nitrogen occurs in the soil not only in the form of nitrates but also as ammonium salts. After the general significance of nitrates as a source of

¹ Belzung, E. Ann. Sci. Nat. (7) 15: 249. 1892; Mazé, P. Bull. Soc. Biol. 8: 98. 1915; and others.

² Schulze, E. Zeitschr. f. physiol. Chem. 22: 82. 1896.

³ Molisch, H. Ber. bot. Ges. 1: 150. 1883; Idem. Sitzungsber. d. Akad. Wien, Math.-natur. Kl. 95: 121. 1887.

nitrogen for seed-plants had become known, the nutritive value of ammonia was underrated for some time.¹ Later it was proved that the ammonia of the soil may serve as a direct nutrient of plants and not have less nutritive value than the nitrates.² The reason for the apparent inequality is as follows.³ Both are taken into the plant as ions since both are completely dissociated in the soil solution on account of their low concentration. The salts of ammonia with strong mineral acids produce an acid reaction in the soil after the ammonia is absorbed by the plant and this acidity is injurious to many plants. Those which do well in acid soils are able to use ammonia as well as nitrates in their nutrition.^b Moreover, many plants which are sensitive to the acid reaction thrive on chalky soils when fed well with ammonia. Therefore ammonia is nearly as good a source of nitrogen for seed-plants as nitrates whenever the plants can tolerate the resultant acid soil reaction or when the soil constituents at once neutralize the acids that are formed. Some green algae in pure cultures with a neutral reaction utilize both ammonia and nitrates⁴ equally well.

Truffaut and Bezsonoff⁵ recently tried to prove that urea excels both ammonium salts and nitrates in nutritive value for seed-plants on all soils. This is probably true, since urea has the following advantages over mineral salts:

1. The plasma membrane of plant cells is very permeable to urea.
2. No acid reaction is produced by the removal of NH_3 from urea.
3. No photochemical reduction is necessary in utilizing urea.

Under natural conditions the soil contains extremely small amounts of ammonium salts and nitrates. It is to be remembered particularly that practically all fixed nitrogen on the earth is of biological origin. Inorganic processes, that is to say the electrical phenomena of the atmosphere, furnish only very minute quantities of fixed nitrogen which mean very little to plants. It is also true that analyses of the rocks of arctic regions indicate only very small amounts of nitrogen in them.⁶ However, in cultivated soils there is a large quantity of nitrogen in the form of proteins and other nitrogenous substances of the protoplasm of living microorganisms.⁷ This conception of soil as a biological complex was at first received with distrust but now it is generally

¹ Knop, W. Landwirt. Versuchs-Stationen. 2: 75. 1860; and others.

² Pitsch und van Lockeren-Campagne. *Ibid.* 34: 217. 1887; Muntz. Compt. rend. 109: 646. 1889; Griffiths, A. B. Chem. News. 64: 147. 1891; Kossowitsch, P. Jour. f. exp. Landwirtschaft, Vol. 5. 1901 (Russian); Mazé. Ann. de l'inst. Pasteur. 14: 26. 1900; cf. especially Schulow, J. Investigations in the field of the nutritional physiology of the seed-plants. 1913 (Russian); The possibility of a formation of nitric acid from ammonia by microorganisms was prevented in the experiments of this investigator.

³ Wagner. Düngungsfragen, Vol. 4. 1900.

^b Some of the factors involved in the relationship of these salts to the plants are brought out in two recent papers by Prianischnikow, D. Ergeb. d. Physiol. 1: 407-446. 1926 and Biochem. Zeitschr. 207: 341-349. 1929. The question is being investigated in many quarters. Friedemann, W. G. (Science 70: 43-44. 1929) has worked out parallel solutions which differ only in the form of the nitrogen which they contain and which are now being tested on cotton plants in Georgia.—Ed.

⁴ Pringsheim, E. G. Beitr. zur Biologie der Pflanzen. 12: 413. 1915.

⁵ Truffaut, G. et Bezsonoff. Compt. rend. 178: 723. 1924; [cf. also Pirschle, K. Biochem. Zeitschr. 212: 466-476. 1929].

⁶ Erdmann. Ber. chem. Ges. 29: 1710. 1906.

⁷ Kostytschew. Jour. f. Land- und Forstwirtschaft for 1890. P. 115 (Russian). Of course there is also a certain amount of organic nitrogen in the humus but the latter is constantly being broken down slowly by various microorganisms. Hence humus appears in the nitrogen cycle of plant and animal residues. Cf. Bréal. Ann. agr. 23: 356. 1897; Stoklasa. Centralbl. f. Bakt. (II) 4: 510. 1898; and others.

recognized since Winogradsky¹ has shown that the quantities of bacteria in soil are many times greater than was previously supposed from inexact estimates. When these organisms die, decay processes change the greater part of their nitrogen and the other constituents of humus into ammonia, through the oxidizing and deaminizing powers of various saprophytic bacteria (*i.e.*, organisms which feed on organic compounds and effect their cleavage or mineralization). This ammonia is either used for its nitrogen by various higher or lower plants or it is oxidized to nitrates. The latter is easily washed into the deeper layers of soil but later it returns to the surface in the soil water which rises by capillarity. In this way, the land plants are able to catch considerable amounts of nitrates. Ammonia, from which nitrates are formed, remains in the soil by adsorption and is very hard to wash out. The relative amounts of organic nitrogen, NH₃ nitrogen and that of nitrates in 1 kg. of soil are shown by the following analyses of Boussingault:²

	Soil 1, g.	Soil 2, g.	Soil 3, g.
Organic nitrogen.....	2.594	5.130	1.397
Ammonia nitrogen.....	0.020	0.060	0.009
Nitrate nitrogen.....	0.175	0.046	0.015

These amounts of ammonia are unusually large; as a rule, ammonia cannot be demonstrated in the soil because it is formed slowly while nitrates are at once formed from it. However, the amounts of nitrates in the soil are always extremely small in comparison with the quantities of organic nitrogen. The following analyses, carried out in the laboratory of the author of this book by N. Lazareff, give additional information concerning the forms of organic nitrogen in various soils.

Forms of N	Podsol soil (North. Russia)		Black soil (Middle Russia)		Loess (Middle Asia)		Red soil (Transcau- casia)		Loam (Palestine)	
	mg.	%	mg.	%	mg.	%	mg.	%	mg.	%
Total N.....	146.5	100	231.5	100	39.2	100	131.7	100	51.2	100
Humus N.....	91.4	62.4	96.9	41.9	12.0	30.7	89.9	68.1	13.0	25.4
Protein N.....	39.4	26.9	95.1	41.1	20.1	51.3	29.9	22.7	31.5	61.6
Residual N.....	13.5	9.2	39.5	17.1	7.0	17.9	10.7	8.1	5.6	10.9

It is a surprising fact that severe rains are able to wash a considerable part of the soil nitrogen (probably living bacteria) into deeper zones of soil.³ For example:

¹ Winogradsky, S. Compt. rend. Acad. Sci. Paris. 177: 1001. 1923; 179: 367. 1924; Ann. de l'inst. Pasteur. 39: 299. 1925; Soil Science. 25: 37. 1928.
² Boussingault. Agronomie, chim. agr. et physiol. 2: 1. 1861.
³ Scheloumow, A. und v. Faermann. Arb. Bur. f. land. Mikrobiologie Leningrad. 3: 8. 1928; Scheloumow, A. und V. Berg. Ibid. 3: 38. 1928.

POROUS LOAM UNDER GRAPE VINE, KRYMSK

N in grams per kilogram of soil (dry weight)

July 13—before the rain.....	1.440
July 14—after the rain.....	0.994

BLACK SOIL (NORTHERN CAUCASIA)

N in grams per kg. of soil

	Uncultivated	Under tobacco	Under sunflowers
July 6—before the rain.....	2.81	2.89	2.93
July 8—after the rain.....	2.69	2.72	2.80

That it is the bacteria that are washed out is shown by the fact that the nitrifying power of the soil is also practically gone after the rain.

The enormous difference between the quantities of inorganic soil nitrogen and the nitrogen found in plants growing on the soil indicates that the plants usually hunger for this important element. We can be sure that there is almost no reserve nitrogen in the soil. The nitrogen made available by the death of organisms is entirely taken up by the living inhabitants of the soil so that each molecule of nitrogen serves to maintain the life of a whole series of generations of animals as well as plants. Clear proof of the accuracy of this conception is to be found in the extremely economical use of nitrogen in plants and in the observation of farmers that nitrogenous fertilizers usually promote the growth of cultivated plants. The exceptions are the *Leguminosae* which are able to assimilate the molecular nitrogen of the air. Thus we have the right to assume that nitrogen hunger is the factor which is most important in limiting the growth of life on earth and in retarding the increase in the number of organisms. However favorable the other living conditions may be for plants, the sum total of living substance cannot exceed a certain amount on account of nitrogen hunger.

It has already been mentioned that phosphorus and sulphur are required for the synthesis of proteins. The details of their assimilation from the soil will be given in Chapter V. Here it is sufficient to point out the following important facts. Numerous growth experiments by different workers have shown that phosphorus and sulphur are available to the seed-plants only as hydrates of their highest oxides, *i.e.*, as salts of orthophosphoric acid and of sulphuric acid.

By using suitable qualitative tests it can be demonstrated that nitrates pass up the vascular tissues of seed-plants as far as the leaves where they are used to form proteins.¹ There have been many changes in the views concerning the use of light in this process. At first the reduction of sulphates and nitrates and the whole process of protein synthesis in seed-plants was regarded as a photochemical reaction.² Then came the idea that light was not used directly

¹ Sachs. Bot. Zeit. 20: 63. 1862; Pagnoul. Ann. Agron. 5: 481. 1879.

² Schimper. Flora. 73: 207. 1890; Idem. Bot. Zeit. 46: 65. 1888; Saposchnikow. *loc. cit.*

in the formation of organic compounds of nitrogen. Some investigators have used exact methods to prove the following facts, already considered by Bous-singault.¹ It appears that seed-plants can synthesize proteins in total darkness by using nitrogen from nitrates if there is enough sugar available at the time.² In complete agreement with this, there are also various observations on organs without chlorophyll³ and on molds which synthesize proteins from nitrates or ammonium salts and sugar without light. From this it is concluded that the reduction of nitrates is not a photochemical reaction and that light affects the synthesis only as it provides sugar.^c

Just recently this conclusion has been disputed.⁴ All the facts given above only prove that the reduction of nitrate is possible at the expense of chemical energy when light is excluded but it is by no means certain that the reduction never uses radiant energy. Some bacteria (see the next chapter) are known to reduce carbonic acid in the dark at the expense of chemical energy but it has also been proved that this reduction is a photochemical process in green plants. The following facts support the idea that nitrate reduction can be directly effected by light. First of all, it must be remembered that nitric acid and nitrous acid can be reduced outside of living cells by rays of short wave length and in the presence of suitable catalysts it goes on fairly easily.⁵ On the other hand, it appears that the synthesis of proteins takes place considerably faster in the light than in the dark⁶ and not all the rays of the solar spectrum are equally active for the process.⁷ Of special importance are the observations on plants grown on sugar solutions in darkness, with microorganisms excluded. The technique of artificial cultures of seed-plants has been carried so far in recent years that it has been possible to run long growth experiments in the presence of organic substances. It was found that seed-plants fed with sugar in darkness can use nitrate nitrogen but it is not utilized as well as ammonia nitrogen.⁸ On the contrary, nitrates are the best source of nitrogen in the light. Thus with respect to their nitrogen nutrition, the seed-plants behave in darkness exactly like the molds which always use ammonia better than nitrates.⁹

Another piece of evidence in favor of the hypothesis that protein synthesis in seed-plants is a direct photochemical process is the deposition of reserve

¹ Boussingault. *Agronomie, chim. agr. et physiol.* 7: 130. 1884.

² Zaleski, W. Conditions for the formation of proteins in plants. 1900 (Russian); Suzuki. *Bull. Coll. Agr. Tokyo.* 2: 409. 1896; 3: 241. 1898; Maliniak. *Rev. gén. bot.* 12: 337. 1900; Warburg, O. und E. Negelein. *Biochem. Zeitschr.* 110: 66. 1920; Muenscher, W. C. *Bot. Gaz.* 75: 240. 1923; Gouwentak, C. A. *Dissert. Amsterdam.* 1929.

³ Ishizuka. *Bull. Coll. Agr. Tokyo.* 2: 471. 1896.

^c This conclusion was also reached by Loehwing from experiments with tomatoes (*Proc. Iowa Acad. Sci.* 34: 115-118. 1927).

⁴ Baudisch, O. und E. Mayer. *Zeitschr. f. physiol. Chem.* 89: 175. 1914; Idem. *Ber. chem. Ges.* 50: 652. 1917; 51: 793. 1918; Moore, B. *Proc. Roy. Soc. London (B)* 90: 158. 1919; Kostytschew, S. und E. Tswetkova. *Zeitschr. f. physiol. Chem.* 111: 171. 1920; Warburg, O. und E. Negelein. *loc. cit.*

⁵ Baudisch, O. und E. Mayer. *loc. cit.*

⁶ Godlewski, E. *Bull. l'acad. sci. Cracovie* 1897 and p. 313, 1903; Warburg, O. und E. Negelein. *loc. cit.*; Chamcowa, M. *Bull. Acad. Pol. Ser. B*, p. 63. 1922.

⁷ Laurent et Marchal. *Rech. sur. la synthèse des subst. albumin. par les végétaux.* 1903.

⁸ Petrow, G. G. The assimilation of nitrogen by seed-plants in the light and in darkness, p. 200. 1917.

⁹ Ritter, G. E. *Materials for the physiology of the molds*, pp. 1-17. 1917 (Russian).

protein, especially in chloroplasts, during photosynthesis.¹ When this simple protein is dissolved out with pepsin and HCl, the chloroplasts shrink in volume. Moreover, various other facts can be clearly explained from the standpoint of photochemical synthesis of proteins. Thus Krascheninnikow and other workers have found that the increase in heat of combustion of leaves after exposure to light always exceeds the value calculated according to the equation for carbohydrate synthesis (p. 100). Perhaps these facts can be explained by saying that proteins as well as carbohydrates are formed at the expense of solar energy. That is, the heat of combustion of protein is greater than that of carbohydrate. Warburg and Negelein obtained in the blue rays an apparently reduced chemical output which was not determined directly but from the equation for the synthesis of sugar. Photochemical synthesis of protein seems to be brought about by the strongly refrangible rays.² For this reason there are objections to the use of the equation of carbohydrate synthesis for experiments in the blue-violet rays.³ It must be remembered in this connection that Timiriazeff (p. 111) obtained no formation of starch in the blue-violet part of the spectrum although the inequality of energy absorption in the red and blue regions is very small. If it is assumed that proteins are formed especially by the blue rays, this result can be understood.

To summarize the foregoing discussion, we have found that the formation of proteins from nitrates in seed-plants is a photochemical process at least in the first stage. With reference to the synthesis of protein at the expense of ammonia nitrogen, it is uncertain whether radiant energy also enters into this process.

The synthesis of protein undoubtedly begins with the formation of amino acids, the structural units of the protein molecule; then these units are united into a complicated molecule. This question will be discussed further in Chap. IV.

16. THE CHEMICAL PROCESSES OF PHOTOSYNTHESIS

It was pointed out in Chap. I that the most important problems of modern chemical plant physiology are summarized in the explanation of the chemistry of material transformations because the chemical exposition of physiological processes is the principal goal of our science. It has also been mentioned that to explain most of the vital processes it is very important to isolate the intermediate products of reactions which consist of several stages and to learn the nature of each of the phases of the reactions.

In photosynthesis—the most important biochemical process of plants—this task is unsolved and there are only a few experimentally established facts. Although we try to avoid in this book all theories which are not based on experiments, the question of the chemistry of important biological processes is an exception to this rule because it is extremely important to work out such

¹ Ullrich, L. *Zeitschr. f. Bot.* 16: 513. 1924.

² Laurent et Marchal. *loc. cit.*

³ With the present condition of our knowledge of photosynthesis, it is entirely inadmissible not to take into consideration the energy absorbed by the yellow pigments.

problems. Because many unproved ideas are often useful for future experimental studies, various hypotheses are here stated and the future will decide which are possible and which are hopeless.

The fundamental chemical problem, the solution of which seems to be most important, is the explanation of the nature of the primary products of photosynthesis, *i.e.*, the organic substances which are formed directly from inorganic materials. Both these first products of photosynthesis (one with and one without nitrogen) are intermediate products in the synthesis of carbohydrates and proteins.

With reference to the intermediate product without nitrogen, there have been several hypotheses proposed but only two of them have been generally received. According to the Liebig hypothesis¹ the first product of photosynthesis is oxalic acid, $\text{COOH}-\text{COOH}$. Baur² has defended this theory in recent times but it hardly appears to be valid. In the first place, it can be pointed out, as contradictory evidence, that the formation of oxalic acid in plants depends on processes of oxidation and not on those of reduction.³ Secondly, the sensitiveness of chlorophyll to an acid reaction should be considered⁴ but, in the third place, it is inconceivable that a carbohydrate could be formed from oxalic acid and the more so because the transformation of carbonic to oxalic acid involves such a slight increase in the potential of energy.

Baeyer's theory,⁵ which supposes formaldehyde CH_2O to be the first product of CO_2 assimilation, has been much more widely considered. It is a favorable sign that this theory is now more than 50 years old and still no directly contradictory facts have been uncovered. Most of the theoretical objections which have been brought out against the Baeyer hypothesis in recent times are based on the point that transformation of carbonic acid to formaldehyde requires too much increase in potential energy and that a photochemical change of carbonic acid only as far as formic acid is much more probable, because this involves a slight increase in potential.⁶ However, this objection is invalid from the biological standpoint. Whatever the primary product may be, it must later become a carbohydrate and an enormous increase in potential from carbonic acid to sugar is *unavoidable in any case* and solar energy is the only means of effecting this increase in potential in living plants. Therefore it is more probable to suppose that carbonic acid is first changed to formic acid and is then reduced to formaldehyde.⁷

Baeyer himself assumes that the first step in photosynthesis is the dissociation of carbon dioxide to form carbon monoxide; the latter is reduced to formaldehyde by the hydrogen of water which is also dissociated. This idea is now regarded as too weak to hold. W. Löb⁸

¹ Liebig, J. v. Liebig's Ann. d. Chem. 46: 58 and 66. 1843.

² Baur, E. Zeitschr. f. physikal. Chem. 63: 683 and 706. 1908; 72: 323 and 336. 1910; Idem. Ber. chem. Ges. 46: 582. 1913.

³ Euler, H. v. Zeitschr. f. physiol. Chem. 59: 122. 1909.

⁴ Willstätter und Stoll. Untersuchungen über Assimilation der Kohlensäure, p. 317. 1918.

⁵ Baeyer, A. v. Ber. chem. Ges. 3: 63. 1870.

⁶ Bredig, G. Die Umschau. 18: 362. 1914; Hoffmann, K. A. und K. Schumpelt. Ber. chem. Ges. 49: 303. 1916.

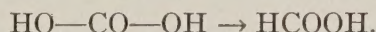
⁷ Kleinstück. Ber. chem. Ges. 51: 108. 1918; Wislicenus. Ibid. 51: 942. 1918.

⁸ Löb, W. Zeitschr. f. Elektrochem. 11: 745. 1905; 12: 282. 1906; Landwirtschaft. Jahrb. 35: 541. 1906.

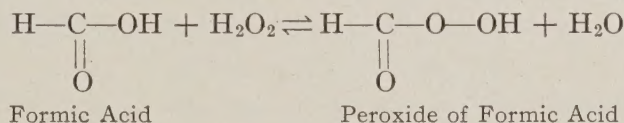
showed that CO_2 and water vapor do dissociate under the action of a silent electrical discharge, the products of which react according to Baeyer's scheme:

1. $\text{CO}_2 + \text{H}_2\text{O} = \text{CO} + \text{H}_2 + \text{O}_2$
2. $\text{CO} + \text{H}_2 = \text{H}_2\text{CO}$
3. $2\text{CO} + 2\text{H}_2 = \text{CH}_2\text{OH}-\text{CHO}$.

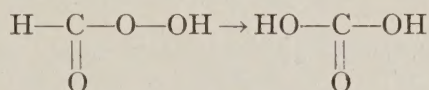
According to Löb's results, then, not only formaldehyde is formed but also glycolic aldehyde, $\text{CH}_2\text{OH}-\text{CHO}$, which has the properties of a sugar. Thus the course of the reaction according to the Baeyer scheme is quite possible and results analogous to those obtained have been given not only by the action of silent discharges but also by the action of light.¹ But every intermediate product of biochemical reactions must respond to tests for its presence and tests for carbon monoxide have all given negative results; it cannot be assimilated by illuminated plants, even in the presence of hydrogen.² Hence it is usually thought to-day that there is no dissociation of carbon dioxide and that in the form of its hydrate H_2CO_3 , it undergoes a direct reduction in the chloroplasts: $\text{H}_2\text{CO}_3 \rightarrow \text{H}_2\text{CO} + \text{O}_2$. Carbon dioxide actually exists in the cells of assimilating tissues in aqueous solution in the form of its hydrate and its reduction is brought about in different cases without the use of radiant energy. Thus magnesium reduces carbonic acid to form formic acid:³



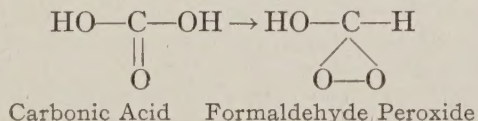
In some cases the reaction is said to go as far as the formation of formaldehyde. This fact is of interest because magnesium is included in the chlorophyll molecule. The reduction of carbonic acid to formic acid also takes place by other methods. Among them the reducing effect of hydrogen peroxide on carbonic acid is especially worthy of attention.⁴ In this case there is probably a formation of a labile organic peroxide according to Manchot's scheme;⁵ this peroxide then decomposes to form a substance which is more easily oxidized than the original substance. These facts agree with the interesting results of d'Ans and his colleagues.⁶ These workers describe the following reaction between formic acid and hydrogen peroxide:



This peroxide of formic acid changes readily into carbonic acid by intramolecular rearrangements:



Willstätter and Stoll⁷ believe that the first step in the photochemical reduction of carbonic acid in the leaf is the transformation of H_2CO_3 into a peroxide-like substance; according to Willstätter and Stoll, the utilization of solar energy is in this process because the subsequent steps involve no increase in potential. Carbonic acid can form not only the foregoing peroxide of formic acid but also an anhydride-like peroxide of the following constitution:



¹ Berthelot, D. et H. Gaudechon. *Compt. rend.* 150: 1690. 1910.

² Just. *Forsch. d. Geb. d. Agr-Physik.* 5: 79. 1882; Richards and McDougal. *Bull. Tor. Bot. Club.* 31: 57. 1904; cf. especially Krascheninnikow, Th. *Rev. gén. bot.* 21: 177. 1909. The contradictory results of Bottomley and Jackson (*Proc. Roy. Soc. London (B)* 72: 130. 1903) are therefore to be regarded as too weak to hold.

³ Fenton. *Jour. Chem. Soc. (London)* 91: 687. 1907.

⁴ Kleinstück. *loc. cit.*

⁵ Manchot. *Liebigs Ann. d. Chem.* 325: 93, 105 and 125. 1902.

⁶ d'Ans, J. und W. Frey. *Ber. chem. Ges.* 45: 1845. 1912; Idem. und A. Kneip. *Ibid.* 48: 1136.

1915.

⁷ Willstätter und Stoll. *Untersuchungen über Assimilation der Kohlensäure*, p. 241. 1918.

This reaction seems to be irreversible whereby the subsequent cleavage of the peroxide must be made easier. The formation of a peroxide in photosynthesis is confirmed by the latest investigations.¹

Carbonic acid was first reduced photochemically by Bach² who used salts of uranium as sensitizers. These results were confirmed by later studies.³ The reduction of carbonic acid has also been effected by ultra-violet light⁴ and by α , β rays.⁵ However, it must be borne in mind that Spoehr⁶ disputes all these results of the action of rays on the basis of his own investigations.

The intermediate products of biochemical reactions can be caught under certain circumstances and they must be worked on by the plants to form the end-products of the particular process (in this case to form carbohydrates). Unfortunately the tests for formaldehyde have not given positive results. Although the literature contains statements which say that formaldehyde has been demonstrated in leaves⁷ (always referred to as small traces), all this information is incorrect. The formaldehyde was either associated with some process other than photosynthesis or demonstrated by means of unsound qualitative methods.⁸ Exhaustive studies have been made by Curtius and Franzen⁹ who distilled enormous quantities of beech leaves without obtaining a definite indication of the formation of formaldehyde during photosynthesis. Mazé¹⁰ also distilled the leaves of various plants at 60°, under reduced pressure and without adding water; he found glycolic aldehyde but no formaldehyde.

It is therefore not yet possible to demonstrate formaldehyde in photosynthesis. Of course, under natural conditions this substance cannot accumulate because it is very toxic. Hence, some method must be used to interfere with the normal course of the entire process by destroying the coördination of the

¹ Warburg, O. und T. Uyesugi. *Biochem. Zeitschr.* 146: 486. 1924; Peklo, J. *Chem. News.* 129: 21. 1924.

² Bach, A. *Arch. des sc. phys. et nat.*, Vol. 5: 1898.

³ Usher, F. L. and J. H. Priestley. *Proc. Roy. Soc. London (B)* 84: 101. 1911; Moore, B. and T. A. Webster. *Ibid.* 87: 163. 1913.

⁴ Usher and Priestley. *loc. cit.*; Berthelot, D. et H. Gaudechon. *loc. cit.*; Stoklasa, J. und W. Zdobnický. *Biochem. Zeitschr.* 30: 433. 1911; Stoklasa, J. Sebor und W. Zdobnický. *Ibid.* 41: 333. 1912; 47: 186. 1912.

⁵ Usher and Priestley. *loc. cit.*

⁶ Spoehr, H. A. *Jour. Amer. Chem. Soc.* 45: 1184. 1923.

⁷ Reinke, J. *Ber. chem. Ges.* 14: 2144. 1881; Mori, A. *Nuovo giorn. bot.* 14: 147. 1882; Pollacci, G. *Atti dell'ist. bot. di Pavia.* 6: 27. 1889; 7: 45. 1899; Grafe, V. *Ost. bot. Zeit.* 56: 289. 1906; Gibson, Ann. Bot. 22: 117. 1908; Schryvers, S. B. *Proc. Roy. Soc. London (B)* 82: 226. 1910; Klein und Werner. *Biochem. Zeitschr.* 168: 361. 1926; [the quantities reported by the latter were 8 to 15 mg. per 10 grams of green leaves but even this small amount is present only when conditions are favorable for photosynthesis although Barton-Wright and Pratt (*Biochem. Jour.* 24: 1210-1216. 1930) say that the action is that of light directly on solutions of CO₂].

⁸ Spoehr, H. A. *Biochem. Zeitschr.* 57: 95. 1913; Willstätter und Stoll. *Untersuchungen über Assimilation der Kohlensäure*, p. 387. 1918; Fincke. *Biochem. Zeitschr.* 51: 253. 1913; 52: 214. 1913.

⁹ Curtius, Th. und H. Franzen. *Ber. chem. Ges.* 45: 1715. 1912; *Idem.* *Sitzungsber. d. Heidelberg Akad. Wiss., Math.-nat. Kl. (A)* 7: 17. 1912; cf. especially *Idem.* *Liebigs Ann. d. Chem.* 404: 93. 1914. The writers determined aldehydes by oxidizing them to acids by means of silver oxide. Formic acid would come from formaldehyde but it also appears in small amounts by the action of the silver oxide on methyl alcohol. Considering the enormous quantity of beech leaves used, the methyl alcohol from the chlorophyll would give some formic acid. This is an instructive illustration of the drawbacks to methods which use large amounts of plant materials to find the intermediate products of biochemical reactions. Unavoidable sources of error are introduced. It should also be mentioned that the work of Curtius and Franzen was done in exemplary fashion with respect to the technique of isolating and identifying new products.

¹⁰ Mazé, P. *Compt. rend.* 171: 1391. 1920.

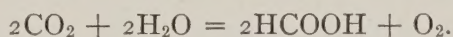
several phases of the process (p. 62). This is the only way by which we can hope to isolate enough of the intermediate product of photosynthesis to mention.

Numerous experiments have been tried to prove that aldehydes can be assimilated by higher and lower plants.¹ Without going into the details of all these investigations, we shall only point out that formaldehyde is usually assimilated in the light. However, this indicates that formaldehyde may not be assimilated directly but must first be oxidized to formic or carbonic acid which is used in the usual way as a plant nutrient. Of course, this does not confirm the formation of formaldehyde in photosynthesis.² Recently it has been shown that plants also assimilate formaldehyde in darkness and thereby increase their dry weight³ but this must be confirmed.

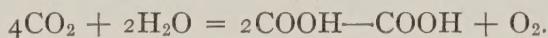
Willstätter and Stoll⁴ noticed an indication of the formation of formaldehyde in photosynthesis in that the ratio of the carbon dioxide absorbed to oxygen released is exactly one,⁵ corresponding to the equation:



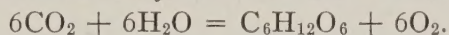
In this equation the molecular amounts of CO_2 and O_2 are equal, as required by Avogadro's law of equal volumes of these gases. The analysis confirms this equality of the gas volumes which can be expressed by the equation $\frac{\text{CO}_2}{\text{O}_2} = 1$. If formic acid were formed in photosynthesis, we should have



In this case $\frac{\text{CO}_2}{\text{O}_2} = 2$, to which the analytical results do not correspond. If oxalic acid were formed, $\frac{\text{CO}_2}{\text{O}_2} = 4$ because



Still the value of $\frac{\text{CO}_2}{\text{O}_2}$ is not indisputable evidence. In photosynthesis it equals 1 not because the CO_2 is changed directly to formaldehyde but because the end product is sugar:



From this equation it is evident that the gas exchange for the entire process gives equal molecular amounts. Whatever the first assimilation product may be, this ratio of the gases must hold after the sugar is made. If formaldehyde were formed, $\frac{\text{CO}_2}{\text{O}_2}$ would have the

correct value from the start but if formic acid should be the first product, the ratio $\frac{\text{CO}_2}{\text{O}_2} = 1$

would come about as follows. In the first stage of photosynthesis $\frac{\text{CO}_2}{\text{O}_2}$ would equal 2 but in the

¹ Bokorny, Th. Ber. bot. Ges. 9: 103. 1891; Idem. Pflügers Arch. f. ges. Physiol. 125: 467. 1908; 128: 565. 1909; Idem. Biochem. Zeitschr. 36: 83. 1911; Grafe, V. Ber. bot. Ges. 29: 19. 1911; Idem. Biochem. Zeitschr. 32: 114. 1911; Treboux. Flora. 92: 49. 1903; Baker. Ann. Bot. 27: 411. 1913; and others.

² Willstätter und Stoll. Untersuchungen über Assimilation der Kohlensäure, pp. 165 and 380. 1918; Nicolas, E. Compt. rend. 175: 1437. 1922.

³ Jacobi, M. Biochem. Zeitschr. 101: 1. 1919; 128: 119. 1922; Sabalitschka, Th. und H. Riesen-berg. Ibid. 144: 545. 1924; 145: 373. 1924; Sabalitschka, Th. Ibid. 148: 370. 1924; Idem. Zeitschr. f. angew. Chem. 35: 684. 1922; [Idem und Weidling. Biochem. Zeitschr. 172: 45-57. 1926; 176: 210-224. 1926; Bodnar, J., L. E. Roth und C. Bernauer. Ibid. 190: 304-325. 1927].

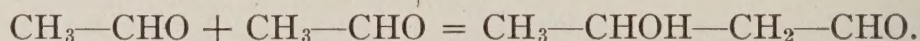
⁴ Willstätter und Stoll. Ber. chem. Ges. 50: 1777. 1917; Idem. Untersuchungen über Assimilation der Kohlensäure, p. 315. 1918.

⁵ Maquenne, L. et E. Demoussy. Nou. rech. sur les échanges gazeux des plantes vertes avec l'atmosphere, p. 115. 1913; Willstätter und Stoll. loc. cit.; Kostytschew. Ber. bot. Ges. 39: 319. 1921.

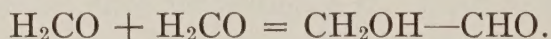
second step it would be 0.5 (when sugar is formed from formic acid). Then the value of 1 for $\frac{\text{CO}_2}{\text{O}_2}$ would be the mean value of 2 and 0.5. So the constant value of $\frac{\text{CO}_2}{\text{O}_2}$ only proves that carbohydrates are practically the only product of photosynthesis.

As yet there has been no clear demonstration of the primary formation of formaldehyde and, as before, the Baeyer hypothesis is not superceded; if no experimental evidence has been obtained to prove it, on the other hand no facts have been reported which directly contradict it. In the mean time it is easy to explain the formation of sugar by assuming that formaldehyde is the primary product of photosynthesis, because this part of the process must take place without the slightest expenditure of energy. When a molecule of glucose is formed from carbonic acid and water there is a gain of 674 Calories. The formation of the corresponding molecular quantity of formaldehyde yields 740 Calories; of formic acid, 370 Calories; of oxalic acid, only about 280 Calories.¹ Hence there is an insufficient increase in the potential when the acids are formed and we must suppose that in this case *there are other photochemical reactions*, or the synthesis of sugar is not explained. On the contrary, if we assume formaldehyde to be the primary product, *photosynthesis is accomplished by a single photochemical reaction*, which is the reduction of carbonic acid or the intramolecular rearrangement of carbonic acid (*cf.* p. 123). This hypothesis is the more probable.^d

Formaldehyde was first condensed to sugars by Butlerow² who used alkalies. According to the current conception, this process is the so-called aldol condensation. Two molecules of aldehyde unite into one molecule by the change of one carbonyl group to a hydroxyl group at the expense of hydrogen of the other molecule of aldehyde. This leaves a free valence in each molecule and they polymerize. For example, 2 molecules of acetaldehyde form one aldol molecule:



The discovery that formaldehyde can also give an aldol condensation³ was an important step toward understanding photosynthesis. Glycolic aldehyde is formed from two molecules of formaldehyde:



Glycolic aldehyde is a sugar-like substance; by aldol condensation with formaldehyde it gives glyceric aldehyde, a triose, which has sharply defined properties of a sugar:



¹ Since the problematical reactions which could lead to all these hypothetical products, cannot be measured exactly, these energy values are only approximate estimates. They are close enough though to give an idea of the situation.

^d By a process called photocatalysis, Baly (Baly, E. C. C. *Ind. Eng. Chem.* 16: 1016-1018. 1924; *Idem.* *Science.* 68: 364-367. 1928; and other papers cited in these general articles) claims to have produced formaldehyde from carbon dioxide and water. By *in vitro* experiments with CO₂, water, quartz vessels and light from a quartz mercury vapor lamp, "active formaldehyde" is synthesized and polymerized to carbohydrates. Baly has also announced the synthesis of carbohydrates by visible light waves which was made possible by the suspension of colloidal particles in the solution of carbon dioxide. The resulting carbohydrates include glucose, fructose and even larger sugars.—*Ed.*

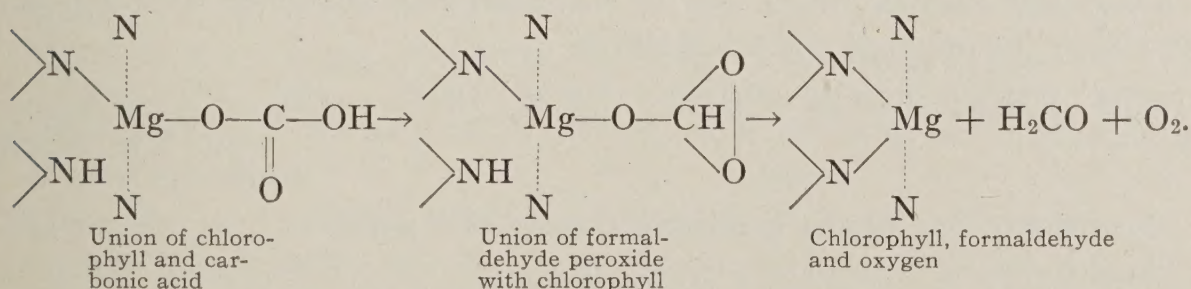
² Butlerow. *Liebigs Ann. d. Chem.* 120: 295. 1861; Confirmation: Loew, O. *Ber. chem. Ges.* 19: 141. 1886; 20: 142 and 3039. 1887.

³ Euler, H. v. und A. v. *Ber. chem. Ges.* 38: 1556. 1905.

The further transformation of trioses to hexoses was done long ago by E. Fischer and his colleagues.¹ Glycolic aldehyde can also be condensed directly to hexoses.² All these condensation reactions take place at a weak alkaline reaction and without any consumption of energy. Hence the change of formaldehyde to sugar is nothing more than the sum of reactions which have already been performed outside of living cells.

The Baeyer theory must be supplemented to explain the asymmetrical synthesis. On the basis of this theory, optically active sugar would be formed from optically inactive formaldehyde while the artificial syntheses outside of living cells have yielded only optically inactive sugars. The fundamental physiological difference between optically active and inactive substances was explained in Chap. I. Although the capacity of living cells and their enzymes to form asymmetrical molecules apparently without the aid of optically active substances is not precluded on the basis of various observations, this does not seem to be a proved fact. In our laboratory practice we can effect asymmetrical syntheses only by using asymmetrical substances.

For this reason the Baeyer theory is now supplemented by the assumption that carbonic acid is reduced while it is united with large asymmetrical molecules. According to this conception, neither does the subsequent condensation of the reduction products take place in the form of free molecules. Some experimental results support this view. It was pointed out above that chlorophyll can form loose unions with carbonic acid and that the leaf tissues also take up CO₂ in the dark. It has now been proved experimentally that the absorption of CO₂ and the release of oxygen by illuminated green leaves are two independent, separate processes. Under certain conditions the oxygen is not liberated until some time after the carbon dioxide is absorbed.³ Willstätter explains this by the change of the chlorophyll-carbonic acid compound to the peroxide form; this peroxide then decomposes and liberates the molecular oxygen. Willstätter and Stoll⁴ believe that this peroxide-like compound splits off two oxygen atoms in succession. The process can be represented as follows:



In this way the action of chlorophyll as a sensitizer becomes more conceivable. The solar energy absorbed by this pigment acts on the union of chlorophyll and carbonic acid. The result is the rearrangement of the carbonic acid into a labile peroxide compound which then readily decomposes to form

¹ Fischer, E. und Tafel. Ber. chem. Ges. 20: 3383. 1887; 21: 2634. 1888.

² Fenton, H. Proc. Chem. Soc. for 1897, p. 63; Fenton and Jackson. Chem. News. 80: 177. 1899.

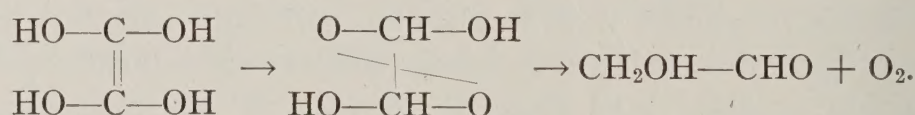
³ Kostytschew. Ber. bot. Ges. 39: 319. 1921.

⁴ Willstätter und Stoll. Untersuchungen über Assimilation der Kohlensäure, p. 244. 1918; cf. also Warburg, O. und Uyesugi. Biochem. Zeitschr. 146: 486. 1924; Peklo, J. Chem. News. 129: 91. 1924.

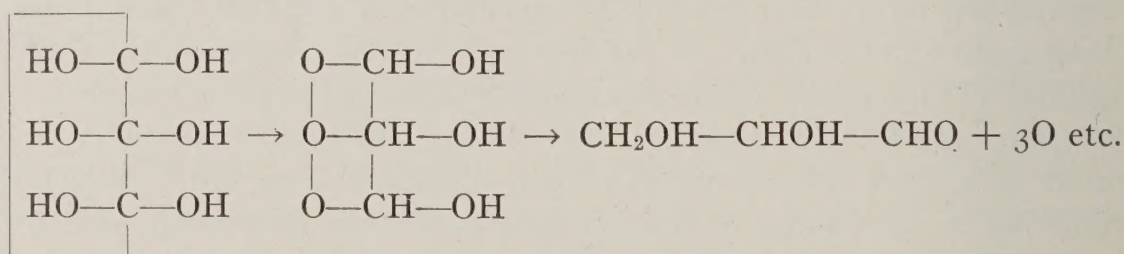
oxygen and formaldehyde. Such an explanation helps to explain the sensitizer effect. The transmission of solar energy to other bodies has been hard to understand from the chemical standpoint. By this scheme, the energy transfer can be traced to intramolecular rearrangements of the very substances which are sensitive to light. This explanation is very simple and easy to understand.

In order to explain asymmetric synthesis, it is usually assumed that the formaldehyde arising from the reduction of the peroxide remains attached to the chlorophyll and undergoes polymerization in this form, or that it joins itself to other complicated asymmetric substances such as proteins.¹ The latter case is conceivable because the further process of forming sugar is exothermal and can thus proceed without the use of chlorophyll, which then fixes another molecule of carbonic acid, etc.

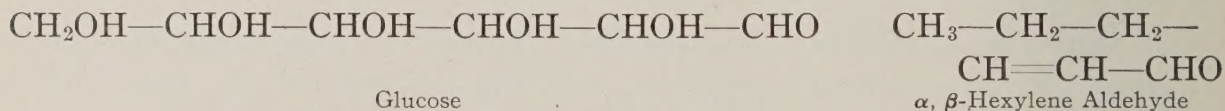
The latest theories of photosynthesis suppose that the unsaturated groups CHOH or C(OH)_2 ,² and not formaldehyde, join together with the help of enzymes, and thus form asymmetric substances. As already explained (p. 62), such theories alone deserve attention. Glycolic aldehyde can be formed directly from two of the group C(OH)_2 :



Three groups can give glyceric aldehyde and oxygen is also liberated.



It has already been mentioned that the formation of formaldehyde has not yet been proved for photosynthesis. According to Curtius and Franzen,³ α , β -hexylene aldehyde, which may be a by-product of the synthesis of glucose, constitutes the principal volatile aldehyde.



Maquenne⁴ develops an analogous theory. He points out that chlorophyll is found in leaves in colloidal solution (*cf.* p. 73). In colloidal solutions there are always aggregates of molecules. Assuming that the complex unions of the chlorophyll molecule come to pass through the agency of the magnesium atom (according to Maquenne, the nature of the complex union is of no importance),

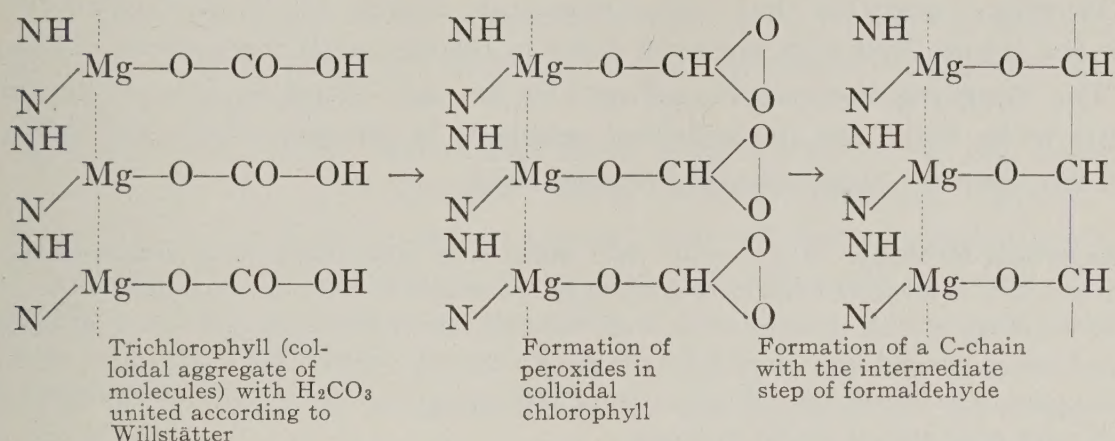
¹ Fischer, E. Ber. chem. Ges. 27: 3189. 1894; Idem. Untersuchungen über Kohlenhydrate und Fermente, p. 111. 1909; *cf.* especially Idem. Jour. Chem. Soc. (London) 91: 1749. 1907.

² Woker, Gertrud. Pflügers Arch. f. ges. Physiol. 176: 11. 1919; Kögel, R. P. Biochem. Zeitschr. 95: 313. 1919; 97: 21. 1919; *cf.* especially Noack, K. Zeitschr. f. Bot. 12: 273. 1920.

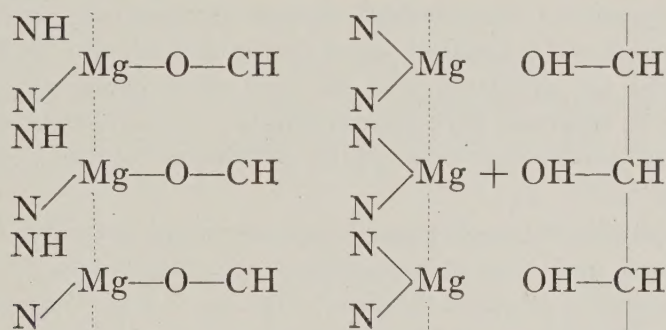
³ Curtius, Th. und H. Franzen. Sitzungsber. d. Heidelberg Akad. Wiss., Math.-nat. Kl. (A) 7: 17. 1912; Idem. Liebigs Ann. d. Chem. 404: 93. 1914.

⁴ Maquenne, L. Bull. de la soc. chim., séance du 4 avril, 1924.

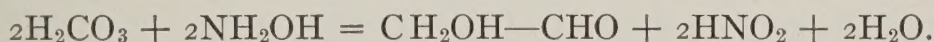
Maquenne writes down the following formulae which are said to explain the synthesis of carbohydrates from carbonic acid:



The resultant carbon chain is finally split off as a sugar and the colloidal chlorophyll is regenerated:



Mazé¹ also failed to obtain formaldehyde in the vacuum distillation of leaves but he was able to isolate glycolic aldehyde. On the basis of this result, he believes that both the synthesis of sugar and the assimilation of nitrogen take place simultaneously by way of the intermediate stage of hydroxylamine. According to Mazé, both nitrates and ammonia become hydroxylamine. A reaction between it and carbonic acid is said to give glycolic aldehyde and nitrous acid, using the energy of light.²



The hydroxylamine acts as a catalyst here and is reformed from the nitrous acid:



The ratio $\frac{\text{CO}_2}{\text{O}_2}$ is 1 for the reaction, thus corresponding to the experimental results.

Wo. Ostwald³ assumes that lipoids play an important rôle in photosynthesis by changing CO_2 into a peroxide. Thunberg⁴ and Weigert⁵ propose very

¹ Mazé, P. Compt. rend. 171: 1391. 1920.

² Mazé, P. Ibid. 172: 173. 1921.

³ Ostwald, Wo. Kolloid-Zeitschr. 33: 356. 1923.

⁴ Thunberg, T. Zeitschr. f. physikal. Chem. 106: 305. 1923.

⁵ Weigert, F. Ibid. 106: 313. 1923.

original theories according to which the photochemical action is on water which is thereby dissociated. It is impossible to consider these theories here.

Wurmser¹ suggests that iron compounds release O₂ in photosynthesis so that the chlorophyll does not even come in contact with the gaseous oxygen.

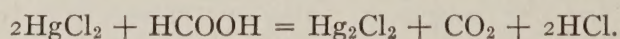
The foregoing theoretical assumptions are not based on facts. The main points to be noted are the unsolved problems in photosynthesis and the wide field left open for experimental investigations.

Analytical Methods.—The various tests which have been proposed to demonstrate very small amounts of formaldehyde have given negative results with all the other aldehydes. One of the latest trials of all these tests² showed that only one of them is reliable; even in the presence of strong mineral acids, formaldehyde gives a red color with Schiff's reagent.³ To 10 cc. of the liquid to be tested, add 2 drops of strong HCl and 4 drops of 1% fuchsin-sulphurous acid. A red color must appear within 24 hours.

If the output of formaldehyde is larger, it may be either oxidized to formic acid by means of alcoholic hydrogen peroxide, or determined as a characteristic derivative of formaldehyde, such as urotropine⁴ N(CH₂—N=CH₂)₃ or p-nitro-phenylhydrazone CH₂=N—NH—C₆H₄—NO₂ (melting point 181–182°).⁵ One element, such as nitrogen, is determined quantitatively in these substances. Formic acid is changed to the zinc or lead salt and the Zn or Pb is measured. If the analytical results are compared with the theoretical percentage of this element, the isolated substance can be identified with more certainty. If formaldehyde is to be changed to an acid, it must first be proved that there are no acids or no methyl alcohol contained in the experimental material.

The quantitative determination of formaldehyde is carried out either by direct titration or by measuring it as formic acid after first oxidizing the formaldehyde (see above). The direct titration is done with iodine in alcoholic solution by the use of a 0.1 M solution of thiosulphate.⁶ In the presence of other aldehydes, the formaldehyde can be determined from the amount of KCN which it takes up because the other aldehydes react with cyanide more slowly.

Formic acid is qualitatively recognized by its capacity to reduce silver salts, by which it differs from all the other volatile acids. It forms characteristic salts with zinc and especially with lead; the latter is only slightly soluble in water. Two methods can be used for quantitative determinations in the presence of other acids. By the first one, the mixture of acids is steam-distilled and the distillate containing the free volatile acids is titrated with 0.1% NaOH, the neutral solution concentrated and then boiled in a reflux condenser with equal amounts of a chromic acid mixture (90 g. K₂Cr₂O₇ and 400 g. conc. H₂SO₄ per liter of solution). The liquid is again distilled by steam and the distillate is titrated. The difference between the first and the second titration gives the data for calculating the quantity of formic acid which was destroyed by the chromic acid mixture.⁷ According to the second method,⁸ the neutral solution is heated on a water bath with pure crystalline sublimate [of mercury] in the presence of sodium acetate. The amount of formic acid is calculated from the amount of sublimate that is reduced to calomel. The insoluble precipitate of calomel is filtered off and weighed dry. Thus the method is based on the aldehyde-like properties of formic acid:



¹ Wurmser, R. Rech. sur l'assimilation chlorophyllienne. 1921.

² Fincke, H. Biochem. Zeitschr. 51: 283. 1913; Willstätter und Stoll. Untersuchungen über Assimilation der Kohlensäure, p. 381. 1918.

³ Denigès. Jour. de phar. et de chim. (6) 4: 193. 1896; Idem. Compt. rend. 150: 529. 1910.

⁴ Legler. Ber. chem. Ges. 16: 1333. 1883; Kippenberger. Zeitschr. f. analyt. Chem. 42: 686. 1903.

⁵ Bamberger. Ber. chem. Ges. 32: 1807. 1899.

⁶ Fresenius und Grünhut. Zeitschr. f. analyt. Chem. 44: 13. 1905.

⁷ Kostytschew, S. an unpublished work.

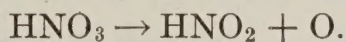
⁸ Franzen, H. und Egger. Jour. f. prakt. Chem. (2) 83: 3213. 1911.

With reference to the assimilation of nitrogen, we must here confine ourselves to the problem of nitrate reduction by green plants and consider the first organic compounds of nitrogen which may be formed in the assimilation of nitrates. Other questions involved in the synthesis of proteins will be considered in Chap. VI.

Baudisch and Mayer¹ found that nitric acid is reduced by the action of both ultraviolet rays and sunlight. More recently,² Baudisch reported that complex ferrous compounds reduce organic nitro compounds and nitrites but not nitrates. He believes that complex ferrous compounds in living cells act as catalysts of the photochemical reduction of nitrogenous compounds containing oxygen. Since the facts about the reduction of nitric acid by short-wave rays has been confirmed in recent times, we are justified in concluding that the photochemical reduction of nitrates in green leaves is also possible. This question was considered above.

The first product of nitrate reduction in green plants seems to be nitrites. They were found in plants long ago.³ The first reports excited the suspicion that bacteria might have taken part in the building of the nitrites⁴ but to-day there is no doubt left because different workers have demonstrated the reduction of nitrates to nitrites in seed-plants under sterile conditions.⁵

On the other hand, it has been found⁶ that nitrites, which in higher concentrations are very toxic to seed-plants, are assimilated very well in dilute (0.05–0.08%) solutions and can be used as nourishment. Thus nitrous acid answers to the description of an intermediate product and we may assume that nitric acid is reduced in plants to nitrous acid which is still an inorganic compound:



However the further fate of the nitrous acid is unknown.

Some writers think that nitrites are reduced to ammonia⁷ but others suspect that there is an assimilation of the oxidized nitrogen. Baudisch⁸ seeks to demonstrate that nitrite is reduced to nitroxyl, NOH, which then unites with the formaldehyde, which is formed in the

¹ Baudisch, O. und E. Mayer. *Zeitschr. f. physiol. Chem.* 89: 175. 1914.

² Baudisch, O. *Ber. chem. Ges.* 50: 652. 1917; 51: 793. 1918; Baudisch und P. Mayer. *Biochem. Zeitschr.* 107: 1. 1920.

³ Laurent, E. *Ann. Inst. Pasteur.* 4: 722. 1890.

⁴ Jorissen, A. *Bull. de l'acad. Belg.* Vol. 13. 1887.

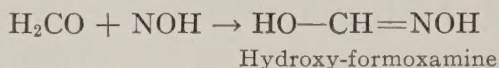
⁵ Godlewski, E. und Polzeniusz. *Bull. de l'acad. Cracovie*, p. 227. 1901; Nabokich. *Ber. bot. Ges.* 21: 398. 1903; Azo. *Beih. z. bot. Centralbl.* 15: 208. 1903; Kastle and Elvove. *Amer. Chem. Jour.* 31: 606. 1904; Mazé. *Ann. Inst. Pasteur.* 25: 289 and 369. 1911; Bach, A. *Biochem. Zeitschr.* 52: 412. 1913.

⁶ Perciabosco e Rosso. *Stat. sperim. agr. ital.* 42: 5. 1909; Mazé. *loc. cit.*; and others.

⁷ Mazé. *loc. cit.*; Loew. *Ber. chem. Ges.* 23: 675. 1890; Jermakoff. *Mitt. d. Univ. Kiew* 5: 1. 1908; and others [including Miss Eckerson (*Bot. Gaz.* 77: 377–390. 1924) who worked with tomatoes containing an abundance of carbohydrates. At certain points in the plants she found a reducing substance which is not inactivated like an enzyme and which takes nitrates as far as ammonia and amino acids also appear in the same tissues. Her conclusion was that "there is in tomato plants an active substance which, on being added to an alkaline solution of potassium nitrate containing fructose or glucose, causes rapid reduction of nitrates to nitrites, and a slower reduction of nitrites to ammonia." Light does not appear to change the rate of the action. From some unpublished work with both tomato and wheat plants, the same investigator has confirmed the results of the earlier work and has learned of other properties of this unknown substance.]

⁸ Baudisch, O. *loc. cit.*; also Baly, E. C. C., J. M. Heilbron and D. P. Hudson. *Jour. Chem. Soc. (London)* 121: 1078. 1922.

light at the same time, to yield hydroxy-formoxamine which, according to Baudisch, is the first organic compound of nitrogen to appear in photosynthesis.



Baly and Stern¹ hold analogous views.

The further transformation of hydroxy-formoxamine to amino acids and other building stones of proteins is not easily explained. Hence it is more probable that either nitrous acid or hydroxylamine reacts with organic substances free from nitrogen.² Actually there seems to be a complete reduction of nitrates and nitrites to ammonia.³

Treub,⁴ has developed entirely different ideas of the assimilation of nitrates. He thinks that hydrocyanic acid, HCN, is the first organic nitrogen compound to be formed by photosynthesis. In the free form HCN is a powerful poison for protoplasm but when fixed in compounds and especially in glucosides it is nearly inactive and accumulates in appreciable amounts under certain conditions, usually in tropical plants but also in some native species.⁵ However, it is almost inconceivable from the chemical standpoint that the cyan group should become an amino group and there has been no satisfactory explanation of this reaction in living plant cells.⁶ Still there has been some evidence of the assimilation of HCN by pure cultures of seed-plants.⁷ It is not improbable that it serves not as an intermediate product of the assimilation of nitrates but in an entirely different but important physiological process, by taking part in the syntheses of carbohydrates and amino acids according to Strecker's scheme.⁸

On the basis of all these facts and considerations, the complete photochemical reduction of nitrates to ammonia is to be regarded as the most probable course in the assimilation of nitrates. Warburg and Negelein⁹ have actually shown that there is a reduction of nitrates to ammonia in the single-celled alga *Chlorella vulgaris*. Smirnow and Erygin¹⁰ did the same for seed-plants. In the section on protein transformations it will be pointed out that it explains all these processes to consider ammonia as the primary and end-product of nitrogen metabolism in plants. This view is supported by various kinds of experimental evidence.

Analytical Methods.—Directions for the determination of nitrates can be found in any book on analytical chemistry.⁹ To make a qualitative test for nitrates in the soil or in plant tissues, the usual procedure is to apply the sensitive test of the effect of a solution of diphenylamine in concentrated sulphuric acid or, better yet, hydrochloric acid (a large amount of reagent must be used for a small quantity of the solution to be tested). A strong blue color develops in the presence of nitrates or nitrites. The most accurate quantitative method for

¹ Baly and Stern. *Ibid.* 123: 185. 1923.

² *loc. cit.*

³ On this point cf. Kostytschew und E. Tswetkova. *Zeitschr. f. physiol. Chem.* 111: 171. 1920; [also Eckerson (*loc. cit.*) and Loehwing. *loc. cit.*].

⁴ Treub, M. *Ann. jard. Buitenzorg.* 4: 86. 1904; 6: 80. 1907.

⁵ Treub, M. *loc. cit.*; Hébert, A. *Ann. agron.* 24: 416. 1898; Dunstan and Henry. *Chem. News.* 85: 301. 1902; Guignard. *Compt. rend.* 141: 1193. 1905; Walter, O., T. Krasnosselsky, N. Maximow and W. Maltshchewsky. *Mitt. d. russ. Akad. d. Wiss. for 1911*, p. 397 (Russian); Henrici, M. *Dir. Vet. Res. Rep.* 11/12: 495. 1927.

⁶ Franzen, H. *Sitzungsber. d. Heidelberg Akad. Wiss., Mathnat. Kl.* 1900; Baly et al. *loc. cit.*

⁷ Korsakowa, M. *Mitt. d. milchwirtschaft. Inst. in Wologda.* 2: 3. 1922; Rosenthaler, L. *Biochem. Zeitschr.* 190: 186. 1927.

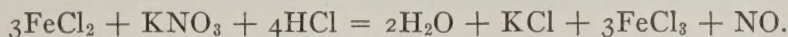
⁸ Euler, H. v. *Grundlagen und Ergebnisse der Pflanzenchemie.* 3: 134. 1909.

⁹ Warburg, O. und E. Negelein. *Biochem. Zeitschr.* 110: 66. 1920.

¹⁰ Smirnow, A. L. and P. S. Erygin. *Russ. Jour. f. Landwirt. Wiss.* 3: 724. 1926 (Russian).

⁹ Methods other than those mentioned here include those described by Pyne, G. T. *Jour. Agr. Sci.* 71: 153-161. 1927; Ranker, E. R. *Ann. Missouri Bot. Gard.* 13: 391-424. 1926; and Sessions, A. C. and J. W. Shive. *Plant Physiol.* 3: 499-511. 1928.—*Ed.*

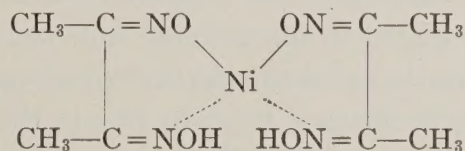
nitrate is that of Schloesing¹ which is used in different modifications sometimes considered to be special methods. The principal of the method is the oxidation of FeCl_2 in acid reaction to FeCl_3 by nitric acid. The quantity of nitric acid is easily calculated from the measurement of nitric oxide released, as shown by the equation.



Another method, which is more convenient to use because of its simplicity, is the use of nitron (diphenylene-diamido-dihydro-triazol) which forms an insoluble compound with nitric acid in weak acid reaction. It is very useful in determining small amounts of nitric acid because the high molecular weight of nitron gives a heavy precipitate, the weight of which is multiplied by the factor 0.168 to give the weight of the nitric acid in it.

A less accurate but rapid and convenient method of determining nitrates is the decoloring of indigo by nitric acid in the presence of an excess of sulphuric acid. To the solution to be tested is added twice its volume of concentrated H_2SO_4 which warms the mixture. Without allowing it to cool, titrate it rapidly from a burette with a suitable solution of indigo (6–8 cc. of indigo solution should correspond to not more than 1 mg. of N_2O_5) until the color changes from blue to green. The first titration is only for orientation; for the second, add most of the required amount of indigo solution at once and then continue to titrate until the color suddenly changes.

Nitrous acid gives the same blue color with diphenylamine dissolved in concentrated HCl . It can also be demonstrated by the following extremely sensitive test. If the liquid to be tested is treated with a mixture of sulphanilic acid [1, 4-aminobenzolsulpho acid, *i.e.*, $\text{C}_6\text{H}_4(\text{NH}_2)(\text{HSO}_3)$] and alpha-naphthylamine ($\text{C}_{10}\text{H}_7\text{—NH}_2$), a red color appears in the presence of very small amounts of nitrites. This test can be used for a colorimetric quantitative determination of nitrites. Hydroxylamine can best be identified by using a modification of the reliable and sensitive dioxyamine reaction of Tschugajew.² This reaction was proposed as an extremely sensitive test for nickel ions and is also used for quantitative determinations of nickel. The principal is the formation of characteristic stable complex compounds (dioxymines) between alpha-keto dioxymines and metals of Mendelejeff's 8th group. Thus dimethylglyoxime, $\text{CH}_3\text{—C}(\text{NOH})\text{—C}(\text{NOH})\text{—CH}_3$, forms with nickel a relatively insoluble, red compound with a typical crystalline form.



The test is thus modified for hydroxylamine.³ The liquid to be analyzed is made slightly alkaline and to it added a trace of a nickel salt. It is then treated with diacetylmonoxime $\text{CH}_3\text{—CO—C}(\text{NOH})\text{=CH}_3$. If hydroxylamine is present a red precipitate is formed because hydroxylamine forms dimethylglyoxime with diacetylmonoxime and the former gives the usual reaction. Inspection of the crystalline precipitate under the microscope avoids all error because the reaction is specific.

Analytical methods for unoxidized compounds of nitrogen are given in Chap. VI.

17. THE INFLUENCE OF EXTERNAL FACTORS ON THE RATE OF PHOTOSYNTHESIS. THE LAW OF THE PHYSIOLOGICAL MINIMUM

Every physiological process is influenced by external factors. The factors which clearly affect photosynthesis include the carbon dioxide content of the

¹ Schloesing. *Ann. d. chim.* (3) 40: 479.

² Tschugajew, L. *Ber. chem. Ges.* 38: 2520. 1905; 39: 2692. 1906; 40: 3489. 1907; *Idem.* *Zeitschr. f. analyt. Chem.* 46: 144. 1905; *Idem.* *Investigations of complex compounds*, p. 59. 1906 (Russian).

³ Kostytschew und E. Tswetkova. *Zeitschr. f. physiol. Chem.* 111: 171. 1920. Still more sensitive but considerably less specific tests are described by Blom, J. *Biochem. Zeitschr.* 194: 385. 1928.

surrounding air (or water), the light intensity and the temperature. Moreover, there is an indirect effect from all external factors which influence the stomatal action because we have already seen (p. 114) that the gas exchange of the leaf is insufficient for photosynthesis when the stomates are closed.

The so-called optimum is extremely characteristic of all physiological processes.¹ For example, it has been observed that the rate of photosynthesis at first increases with rise in temperature up to a certain point beyond which further increase in temperature has the opposite effect and the rate of the process decreases. If this phenomenon be plotted as a curve, with the degrees of temperature for abscissae and the rates of the physiological process, such as

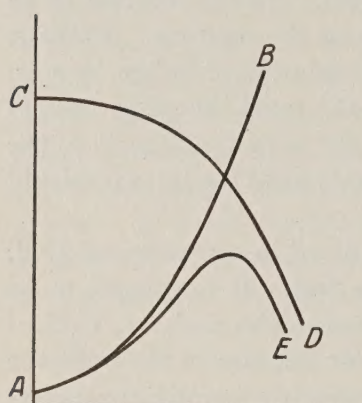


FIG. 19.—Analysis of the optimum curve. Curve *AE* shows an optimum. Actually this is the resultant of curves *AB* and *CD*, the first of which increases continually and the second falls away constantly. (After Blackman.)

the CO_2 absorbed per hour during photosynthesis, for ordinates, the result is a curve of the form *AE* (Fig. 19). It bends toward the axis of abscissae on both sides of the high point.

According to Blackman and his colleagues,² this form of curve depends on the fact that it is the resultant of two or more curves, each of which has a certain form but does not have an optimum. In the case before us, the curve *AE* expresses the effects of two factors, the separate effects of which are shown by the curves *AB* and *CD*, which go in opposite directions. The point at which they intersect corresponds to the "optimum" for the curve *AE*. It is evident from this analysis that there can be no objective optimum. A curve with an optimum point corresponds to the trajectory of a moving projectile, which depends on several factors, the most important of

which are the inertia of motion and the gravitational pull of the earth. Inertia tends to make the projectile fly in a straight line in the original direction while gravity pulls it toward the ground. The resultant determines the trajectory. The point on this path at which the projectile is farthest from the earth's surface corresponds to the optimum of the physiological curve.

Hence Blackman³ has made use of Liebig's law of limiting factors to explain the effect of the surrounding medium on photosynthesis. According to this law, the rate of photosynthesis is determined by the factor present in minimum amount. For example, if the CO_2 content is at a minimum, the rate of photosynthesis is accelerated neither by increasing the light intensity nor by raising the temperature, as long as the CO_2 content remains unchanged.

This rule has not been confirmed by various other investigators with reference to the three most important external factors— CO_2 content, light intensity and

¹ Sachs, J. *Jahrb. wiss. Bot.* 2: 338. 1860.

² Blackman, F. F. *Ann. Bot.* 19: 281. 1905; Blackman and Matthaei. *Proc. Roy. Soc. London (B)* 76: 402. 1905; Matthaei, G. L. C. *Phil. Trans. Roy. Soc. London (B)* 197: 47. 1904; Blackman and A. M. Smith. *Proc. Roy. Soc. London (B)* 85: 389. 1911.

³ Blackman, F. F. *loc. cit.*

heat—and repeated efforts have been made¹ to give a different interpretation to the theory of limiting factors. Just recently, however, Tschesnokow and Bazyrina² are showing that the state of the case has so far been misunderstood. Each limiting factor can act either directly or indirectly. Only in the first case is Liebig's statement correct and self-evident. In the absence of CO₂ or light of course photosynthesis cannot go under any circumstances. In the case of indirect effect, the external factor does not alter the photosynthesis directly but another internal *direct* factor which is at a minimum. Now this direct internal factor may be influenced simultaneously by various indirect factors; in other words, the latter may counteract each other which shows that they act indirectly and are not at a minimum.

Under natural conditions, photosynthesis is influenced chiefly by concentration of CO₂, light intensity and temperature. Of these factors, only light can be at a minimum under certain conditions. However, in almost every case the light acts as a stimulus and therefore indirectly. Both the other external factors can never, under natural conditions, be at a minimum with respect to the photochemical reaction in the true sense of the word.

According to Tschesnokow and Bazyrina, in most cases the rate of transportation of CO₂ to the chloroplasts acts as a direct limiting factor, in which the permeability of the protoplasm to carbonic acid plays an important rôle. This can likewise be influenced by change of either light intensity or concentration of CO₂.

Of course, Blackman's law can be neither disproved nor confirmed by study of the indirect factors. More of the direct factors must be discovered and studied.

The Percentage of Carbonic Acid.—That this is not a direct limiting factor is to be seen from the behavior of plants in natural habitats. For example, more than half the daily output of the leaf may be produced in one hour.³ Many plants of warm habitats keep their stomates open only during the first hours of the forenoon but the crop is not thereby reduced.

That photosynthesis is often accelerated by increasing the concentration of CO₂, as shown by the experiments of several investigators,⁴ only shows that the normal concentration of CO₂ in the atmosphere is not optimum. Moreover, in some cases an increased supply of CO₂ actually works as a stimulant.

The Percentage of Oxygen.—Boussingault found that photosynthesis was not retarded by excluding oxygen and later results have confirmed this observation. Of course oxygen appears the instant that the light rays strike the leaf^f but if

¹ On this point cf. Brown, W. H. Philippine Jour. Sci. (Bot.) 13: 345. 1918; Brown, W. H. and Heise. *Ibid.* 12: 1. 1917; Benecke, W. Zeitschr. f. Bot. 13: 417. 1921; Harder, R. Jahrb. wiss. Bot. 60: 531. 1921; Lundegårdh, H. Biol. Centralbl. 42: 337. 1922; Idem. Der Kreislauf der Kohlensäure in der Natur. 1924.

² This paper is in press.

³ Kostytschew, S., K. Bazyrina und W. Tschesnokow. Planta. 5: 696. 1928.

⁴ Brown, H. T. and F. Escombe. Proc. Roy. Soc. London (B) 70: 397. 1902; Lundegårdh. *loc. cit.*; and others.

^f Harvey's recent paper (Plant Physiol. 3: 85-89. 1928) is an excellent proof of this point. Using marine algae in water containing no O₂, the latter was found to appear within a second.—Ed.

the plant is left for a long time in an atmosphere free from oxygen it loses temporarily the capacity to assimilate. Still the power to assimilate usually reappears soon under normal conditions.¹ It is necessary to distinguish between asphyxiation of the protoplasm (and chloroplasts) and the effect of oxygen on photosynthesis. The experiments just cited dealt with asphyxiation of protoplasm but some experiments do show that oxygen is actually necessary for photosynthesis. In living cells there are almost always some labile compounds of oxygen but they do not occur in chloroplasts² and photosynthesis can therefore be tried in an atmosphere without oxygen. The rate of photosynthesis falls with a very high oxygen tension.³

The Light Intensity.—Boussingault showed that photosynthesis starts instantly in the light and stops just as fast in darkness. The after-effect of light assumed by some authors, *i.e.*, continued, photosynthesis after removal to darkness,⁴ is not confirmed by the more recent studies.⁵ It is very interesting to know the minimum amount of light required to give a perceptible assimilation of CO₂. Ingen-Housz noticed that photosynthesis did not go on in heavy shadows. He also showed that moonlight is not sufficient for the assimilation of carbonic acid but the twilight does give a perceptible assimilation.⁶ The latest studies also show that photosynthesis even at a latitude of 60° is not entirely stopped on a bright summer's night.⁷ Of course, it continues during the night above the Arctic circle.⁸ The large daily output of photosynthesis in northern regions is probably connected with the shortening of the period of growth of some cultivated plants.⁹ All the foregoing facts are barely quantitative in nature. For example, if the normal development of a plant is retarded by shade or when many plants grown in a room gradually show a lack of light, it does not mean that they cannot assimilate under those conditions but that their photosynthesis is not sufficient to meet their vital requirements. The minimum strength of light by which photosynthesis is still possible has been sought by the use of gasometric measurements¹⁰ but this method is not sensitive enough. Only by using phosphorescent bacteria as a test for oxygen has it been possible to show that the minimum intensity is the same for all plants and corresponds to a brightness of 20 meter candles.¹¹

When the intensity of light is increased, the rate of photosynthesis increases rapidly but the curves of the dependence of photosynthesis on light intensity

¹ Boussingault. *Agron., chim. agr. et physiol.* 4: 329. 1868; Pringsheim, N. *Sitzungsber. d. preuss. Akad. d. Wiss.* for 1887. P. 763.

² Willstätter und Stoll. *Untersuchungen über Assimilation der Kohlensäure*, p. 344. 1918; Ewart, A. J. *Jour. Linnean Soc.* 31: 364. 1896 and 32: 554. 1897.

³ Warburg, O. *Biochem. Zeitschr.* 103: 188. 1920.

⁴ Tswett, M. *Zeitschr. f. physikal. Chem.* 76: 413. 1911.

⁵ Richter, A. v. *Mitt. russ. Akad. Wiss.* for 1914. P. 857 (Russian); but *cf.* Warburg, O. *Biochem. Zeitschr.* 103: 188. 1920; This investigator determined the rate of photosynthesis from the oxygen released; on this point *cf.* p. 127.

⁶ Pfeffer, W. *Pflanzenphysiologie*. 2nd ed. 1: 323. 1897.

⁷ Kostyschew, S. *Ber. bot. Ges.* 39: 334. 1921.

⁸ Curtel, G. *Rev. gén. bot.* 2: 7. 1890.

⁹ Kostytschew, S., K. Bazyrina and W. Tschesnokow. An unpublished paper.

¹⁰ Lubimenko, W. *Arb. Petersburg Ges. d. Naturforsch.* Vol. 41. 1910 (Russian).

¹¹ Richter, A. v. and M. Kollegorskaja. *Mitt. russ. Akad. Wiss.* for 1915. P. 457 (Russian)

were very different in the case of the older writers,¹ since light intensity usually acts indirectly. That even in diffuse light of low intensity there is no lack of radiant energy is evident from the fact that increasing the light intensity rapidly lowers the output of photosynthesis.² The phenomena of adaptation also play an important rôle in the action of light.³

Under natural conditions, two categories of plants can be found—sun plants and shade plants. The two groups are adapted to different intensities of light but this adaptation is a complicated phenomenon which influences the whole structure of the leaf. In sun plants there is a particularly strong development of the palisade parenchyma. In the shade plants the epidermis often forms lens-shaped outgrowths and papillae which concentrate the light. Oftentimes these contrivances are so pronounced that the plant shines in diffuse light, as in the protonema of the moss *Shistosiega*.³ In addition, the shade plants have more chlorophyll than sun plants.⁴

Temperature Effects.—An increase in temperature accelerates all chemical reactions but the effect is proportionally small for the process of photosynthesis (p. 68). Every reaction has its limiting temperature below which it does not take place. For photosynthesis the lower limit of temperature varies with different plants. In some tropical plants there is no photosynthesis even at $+5^{\circ}\text{C}.$;⁵ for our native plants the minimum temperature averages $0^{\circ}\text{C}.$;⁶ for many needle-bearing trees there is photosynthesis at very low temperatures. For example, *Picea excelsa* functions at $-35^{\circ}\text{C}.$ and *Juniperus* at $-40^{\circ}\text{C}.$ ⁷ Recent exact studies gave a minimum of $-6^{\circ}\text{C}.$ for *Prunus Laurocerasus*.⁸ Water plants are said to be characterized by a high minimum temperature. *Vallisneria* stops giving off oxygen at $+6^{\circ}\text{C}.$ ⁹ and *Potamogeton* stops at $+10^{\circ}\text{C}.$ ¹⁰ These results need to be confirmed.¹¹ The existence of an optimum temperature¹² is disproved by the latest investigations. The dependence of photosynthesis on temperature is a complicated irritant phenomenon. There can be no question of a direct limiting effect of low temperatures on the photochemical reaction.

¹Wolkow. Jahrb. wiss. Bot. 5: 1. 1866; Kreusler. Landwirt. Jahrb. 14: 913. 1885; Reinke, J. Bot. Zeit. 41: 697. 1883; Peyrou, J. Compt. rend. 105: 385. 1887; Timiriazeff, K. Ibid. 109: 379. 1889; Pantanelli, E. Jahrb. wiss. Bot. 39: 167. 1903; Treboux. Flora. 92: 49. 1903; Blackman and Smith. Proc. Roy. Soc. London (B) 83: 403. 1911; Lubimenko. Rev. gén. bot. 17: 381. 1905; 20: 162. 1908.

²Warburg, O. und E. Negelein. Zeitschr. f. Elektrochem. 102: 235. 1922; 106: 191. 1923; Wurmser, R. Rech. sur l'assimilation chlorophyll. 1921; Puriewitsch, K. Jahrb. wiss. Bot. 53: 210. 1913.

³Some recent experiments on the effect of change of light intensity are reported by Li. Ann. Bot. 43: 587-601. 1929 but the bubble method was used for measurements.—Ed.

⁴Noll. Arb. bot. Inst. zu Würzburg. 3: 477. 1888.

⁵Lubimenko. Compt. rend. 145: 1347. 1907; Idem. The chlorophyll content of chloroplasts and the intensity of photosynthesis. 1910 (Russian).

⁶Ewart. Jour. Linnean Soc. 31: 400. 1896.

⁷Boussingault. Agron., chim. agr. et physiol. 5: 16. 1874.

⁸Jumelle. Compt. rend. 112: 1462. 1891.

⁹Matthaei, G. Phil. Trans. Roy. Soc. London (B) 197: 47. 1904.

¹⁰Sachs, J. Experimentalphysiologie, p. 55. 1865.

¹¹Cloez und Gralivlet. Flora, p. 750. 1851.

¹²Elodea assimilates CO_2 at 7° ; cf. Blackman and Smith. Proc. Roy. Soc. London (B) 83: 400. 1911.

¹³The optimum temperature was agreed upon by all workers until Blackman and his co-workers' work appeared. Cf. especially Kreusler. Landwirt. Jahrb. 19: 649. 1890.

To study the effect of temperature on photosynthesis it is necessary to consider the actual overheating of leaves by direct sunlight, especially when using closed glass containers.¹ It is very possible that the inactivation of chloroplasts² is largely a result of excessive heating of leaves. The inactivation consists in the gradual decrease in the rate of photosynthesis in leaves removed from the plant and exposed to direct sunlight. Finally the process stops although no pathological changes in the leaf can be noticed. After remaining in darkness for some time, the chloroplasts recover and again carry on photosynthesis in the light. Perhaps this process also involves movements of the products of assimilation.

Under certain conditions, chloroplasts are inactivated by a weak ray of sunlight. Serious consequences often come from an excessive insolation in southern latitudes, especially to leaves of cultivated plants which have been brought from a more favorable climate. The author of this book noticed a rapid killing of grapes after an unusually marked insolation under certain meteorological conditions. The leaves wilted fast, turned yellow in the middle of the summer and fell off. This also happened to the young sprouts of the current years growth. Exact measurements show that the leaf temperature can rise to 44° or even to 51° under the influence of direct sunlight,³ after which the leaves wither and die. At noon, Blackman and Matthaei observed the following differences in temperature between leaves and the air:

Experimental Conditions	Rise of Temp. in Leaves
Leaf placed vertically.....	+8° to +7°
Leaf placed at right angles to sunlight.....	+13° to +9°
The same but in a glass container.....	+20°
The same in a glass container behind a water cell.....	+10°

Blackman correctly states that the time factor must be reckoned with in the influence of temperature on photosynthesis because, owing to the progressive inactivation of chloroplasts, the effect of temperature cannot be the same in experiments of different lengths. It is particularly interesting to notice the value of the temperature quotient *i.e.*, the ratio between the increase in temperature and the rate of photosynthesis. It has been pointed out that the reaction velocity of a reaction involving organic substances is doubled or tripled for each 10° interval. Larger increases have been observed in a few cases. Photochemical reactions are different. Their velocity increases slowly as the temperature is raised. A different result is obtained in photosynthesis.⁴ This is probably due to the complicated nature of the process of CO₂ assimilation; in addition to the true photochemical reaction, there are also important reactions which do not depend on light. As a rule, the more recent measurements have shown that the value of the temperature quotient lies midway between those for photochemical and other processes.⁵

¹ Darwin, Fr. Bot. Gaz. 37: 81. 1904; Matthaei, G. *loc. cit.*; Blackman, F. F. and G. Matthaei. Proc. Roy. Soc. London (B) 76: 406. 1905.

² Ewart. Jour. Linnean Soc. 31: 364. 1896; Pantanelli. *loc. cit.*

³ Blackman, F. F. and G. Matthaei. Proc. Roy. Soc. London (B) 76: 407. 1905.

⁴ Matthaei, G. Phil. Trans. Roy. Soc. London. 197: 47. 1904; Blackman, F. F. Ann. Bot. 19: 281. 1905; Smith. Proc. Phil. Soc. Cambridge. 14: 296. 1907; Kanitz. Zeitschr. f. Elektrochem. 11: 689. 1905; Kreusler. Landwirt. Jahrb. 16: 711. 1887.

⁵ Willstätter und Stoll. Untersuchungen über Assimilation der Kohlensäure, p. 152. 1918; *cf.* also Brown and Heise. Philippine Jour. of Sci. (Bot.) 12: 1. 1917; [Osterhout, W. J. V. and A. R. C. Haas. J. Gen. Physiol. 1: 295-298. 1918-1919].

Plant	Temperature Coefficient	
	15°-25°	20°-30°
Yellow Elm.....	1.30	1.07
Green Elm.....	1.48	1.29
Elder.....	1.52	1.45

O. Warburg¹ found very high temperature coefficients, lying between 4 and 5, especially *at low temperatures*. However, we are not really dealing here with an effect of temperature on photosynthesis, because the whole vital activity of the leaf is strongly affected at low temperatures.

The influence of poisons on photosynthesis has already been considered. Poisons retard photosynthesis and produce no preliminary acceleration of CO₂ assimilation. Mechanical stimulation (wounding) has an analogous effect. The curve of the effect of toxic substances is analogous to the isotherm of adsorption.²

The Influence of Water on Photosynthesis.—Water is one of the most important materials for the synthesis of organic substances but before its lack can retard the chemical work of the chloroplasts, it affects photosynthesis indirectly by causing the stomates to close. It has been shown by direct experiments that the CO₂ assimilation of seed-plants³ and of mosses⁴ is considerably reduced by shortage of water. According to the latest studies, there is a limiting value of moisture below which photosynthesis is not possible in seed-plants, even after removing the epidermis.⁵ It is very interesting that various mosses can be entirely dehydrated and yet carry on CO₂ assimilation again after enough water has been replaced.⁶

The Influence of Salts on Photosynthesis.—Photosynthesis is appreciably retarded by lack of the most important ash constituents, namely K, Mg, Fe and P.⁷ For plants which do not grow in a soil containing much salt, treatment with concentrated salt solutions closes the stomates and stops photosynthesis. According to some investigators, salt effect is a complicated phenomenon which does not act in a special way to close the stomates but exerts a direct action on the protoplasm.⁸

The Influence of the Age of the Leaf.—Very young leaves assimilate less vigorously than growing leaves but as soon as the leaf has fully developed, the rate of its assimilation of CO₂ remains constant for unchanging conditions.⁹ As the leaves turn yellow in autumn, the rate of photosynthesis decreases. Green leaves touched by the autumn frost also show a slower rate of photo-

¹ Warburg, O. Biochem. Zeitschr. 100: 230. 1919.

² Warburg, O. Biochem. Zeitschr. 103: 188. 1920.

³ Kreusler. Landwirt. Jahrb. 14: 913. 1885; Déhérain et Maquenne. Compt. rend. 103: 167. 1886; Schroeder, H. und Horn. Biochem. Zeitschr. 130: 165. 1922; Iljin, W. Flora. 16: 360 and 379. 1923.

⁴ Jönsson. Compt. rend. 119: 440. 1894.

⁵ Brilliant, W. Ibid. 178: 2122. 1924.

⁶ Bastit. Rev. gén. bot. 3: 521. 1891; Jumelle. Ibid. 4: 168. 1892.

⁷ Briggs, G. F. Proc. Roy. Soc. London (B) 94: 20. 1922.

⁸ Pantanelli. Jahrb. wiss. Bot. 39: 199. 1903.

⁹ Willstätter und Stoll. Untersuchungen über Assimilation der Kohlensäure, p. 86. 1918. [For reasons stated by Spoeher (Photosynthesis, p. 174. 1926), however, it is still uncertain that newly formed leaves do not have a high capacity for photosynthesis.]

synthesis, in spite of the normal content of chlorophyll. After these inactive leaves have been kept warm for some time they recover their capacity for photosynthesis.¹ In this case and in those described above, the chlorophyll is not the limiting factor.² A case in which the rate is limited by lack of chlorophyll has not yet been found. The dependence of the rate of photosynthesis upon the age of leaves in the spring cannot be attributed to insufficient amounts of chlorophyll.^h

18. THE RATE AND THE DAILY PROGRESS OF PHOTOSYNTHESIS UNDER NATURAL CONDITIONS. ITS GEOCHEMICAL RÔLE

Sachs³ was the first to try to measure the rate of photosynthesis under natural conditions, using his half-leaf method (p. 93). He obtained the following results: taking into consideration the withdrawal of substance from the leaf, the maximum value for assimilation per square meter of leaf surface per hour is 1.8 g. for *Helianthus annuus* and 1.5 g. for *Cucurbito Pepo*.

The first experiments in a current of air were carried out by the great agricultural chemist, Boussingault.⁴ At first his goal was qualitative; he tried to solve the question as to whether the CO₂ of the air can be assimilated by plants. Then Kreusler⁵ used the method for quantitative investigations. The method of moving air currents underwent a further development in the hands of Brown and Escombe.⁶ In their experiments they obtained yields which were three to five times less than those of Sachs. Figures of the same size also appeared in the work of Giltay and Boysen-Jensen.⁷ At first glance it seems that the higher figures of Sachs are attributable to the unavoidable sources of error of his method.

Kostychev and his co-workers⁸ have recently found that the movement of air in the experiments of all the foregoing workers was too slow and the experimental plants suffered from lack of CO₂ at the maximum rate of assimilation.⁹ When the experimental leaves are provided with more CO₂ the rate of movement of the current of air must be at least 1 liter per square centimeter of leaf

¹ Ewart, A. J. Jour. Linnean Soc. 31: 364. 1895; Willstätter und Stoll. Untersuchungen über Assimilation der Kohlensäure, p. 101. 1918.

² Briggs, G. E. Proc. Roy. Soc. London (B) 91: 249. 1920.

^h The question of the influence of electricity on photosynthesis has been raised for many years and investigated by several methods of attack. It was reported by Thouvenin (Rev. gén. bot. 8: 432. 1896), Pollacci (Atti. Ist. Bot. Pavia (2) Vol. 13. 1907) and others that very small electric currents increase the rate of photosynthesis but these results are contradicted by those of Marx, D. Ann. Bot. 43: 163-172. 1929 which indicated no increase and often no change.

Studies of the effect of ionized air on photosynthesis have been made by Henrici (Arch. Sci. Phy. Nat. (5) 3: 276. 1921), Spoehr, H. A. (Bot. Gaz. 59: 366. 1915) and Whimster (Ann. Bot. 41: 357. 1927). All of these workers found an increase in the rate when the green tissues were surrounded with such air but the action is probably on the carbon dioxide rather than on the apparatus for photosynthesis. —Ed.

³ Sachs, J. Arb. bot. Inst. Würzburg. 9: 1. 1884.

⁴ Boussingault, J. B. Economie Rurale, vol. I. 1841.

⁵ Kreusler, M. Landwirt. Jahrb. 14: 913. 1885; 16: 711. 1887.

⁶ Brown, H. T. and F. Escombe. Proc. Roy. Soc. London (B) 76: 29. 1905.

⁷ Giltay, E. Ann. Jard. bot. Buitensorg. 15: 43. 1898; Boysen-Jensen. Bot. Tiddscr. (Kopenhagen) 36: 219. 1918.

⁸ Kostytschew, S., K. Bazyrina und K. Wassilieff. Biochem. Zeitschr. 182: 79. 1927; K., K. Bazyrina und W. Tschesnokow. Planta. 5: 696. 1928.

⁹ In the experiments of Boysen-Jensen (loc. cit.) for example, the air which came out of the leaf chamber was almost entirely free from CO₂.

surface per hour. When these conditions were met, maximum yields were obtained which are entirely analogous to those of Sachs. Sachs' figures are therefore unexaggerated but it would still be wrong to claim that the plant works continually at such a fast rate. Determinations of photosynthesis of various plants at different latitudes and under various climatic conditions, carried out for the entire day, lead to the conclusion that the daily course of photosynthesis is extremely irregular with the maximum yields coming in the morning hours. It often happens that more than half the output for the day is produced in half an hour.

The daily course of photosynthesis is represented by a curve with either one or two maxima.¹ Photosynthesis is often entirely suspended or even replaced by the release of considerable CO₂ in full light,² under entirely favorable external circumstances. The release of CO₂ is not a pathological phenomenon because it lasts only a short time after which normal photosynthesis is resumed. This abundant and short-lived liberation of CO₂ often has nothing to do with respiration. Especially large amounts of CO₂ are liberated by the plants of the deserts of Central Asia.³

The effect of illumination and other meteorological factors in these long-time experiments takes the form of distinct stimulants, with after-effects, etc. The closing of the stomates is often not the cause but the result of the cessation of photosynthesis with full illumination. The leaf in direct sunlight not infrequently works in a way which depends on whether the previous day has been dull or bright.

On the basis of these results it is clear that short experiments with detached leaves are quite unsuited to the study of the ecology of photosynthesis, because the previous history of the leaf is thereby unknown and even the rate of photosynthesis at the moment of removal of the leaf is an unknown quantity. All our present knowledge of the ecology of photosynthesis therefore rests on an extremely uncertain foundation.

The daily yields fluctuate considerably for the same plant. On the average, they are larger in southern (35°–40°) lands than in Middle Russia.⁴ It is a remarkable condition that a given plant on the coast of the polar sea at an average temperature of 5°–12° and with a non-setting sun gives larger daily outputs than in the temperate zone at an average temperature of 20°–25° at the same time of year.⁵ The shortening of the growing season of some crop plants in the arctic climate may depend on this.

It is therefore evident that we do not yet possess sufficient facts for a quantitative estimate of the geochemical significance of photosynthesis.

¹ Kostytschew, Bazyrina and Tschesnokow. *loc. cit.*; Zemtschuznikow und Skaskin. Arb. d. nordkaukas. Association d. wiss. Inst. No. 28, H. 7. 1928.

² Kostytschew, Bazyrina and Tschesnokow. *loc. cit.*; Zemtschuznikow and Skaskin. *loc. cit.*; Wotschal, E. III Kong. d. russ. Bot. 1928; Richter, A. v. an unpublished paper; [Boonstra. *Planta*. 10: 108. 1930].

³ Kostytschew, S. and E. Kardo-Syssoiewa. An unpublished paper.

⁴ *loc. cit.*

⁵ Kostytschew, Bazyrina and Tschesnokow. An unpublished paper.

Nevertheless there have been numerous experiments made to calculate the organic substance formed annually on the earth. Sachs made the following estimate from figures obtained with sunflowers. A healthy sunflower plant stores up 36 g. of dry substance on a sunny day. If this number is taken as a maximum, the average amount for each sunflower is 400 g. per month. If it assumed further that every square meter of the earth's surface produces 400 grams per month,¹ it is easy to calculate that the vegetation of the earth uses 54×10^{12} kg. of carbon dioxide every month. On the basis of the data of Kostychev and his colleagues (see above) the following calculations can be made. The average yield (mean value of several experiments) is about 4.5 grams of carbohydrate per day per square meter of leaf surface. Assuming that one square meter of the earth's surface corresponds to 3 square meters of leaf surface (which is hardly an exaggeration), the monthly production of 405 g. of carbohydrates per square meter of the earth's surface gives the same figure which Sachs found. A. Mayer² believes that each cultivated plant produces about the same amount of organic substance per hectare [= 2.471 acres] per year; for example,

Wheat.....	7410 kg.	Corn.....	7200 kg.
Sugar beet.....	7800 kg.	Beech.....	6922 kg.
Clover.....	7800 kg.	Pine.....	6750 kg.
Meadow hay.....	7200 kg.		

Other computations³ give figures of another order and which differ greatly. Thus Becquerel reported the following annual yields of organic substance per hectare:

Woodland.....	1,800-3,000 kg.
Meadow.....	3,500-4,500 kg.
Cultivated plants in our latitude.....	6,000-9,000 kg.
Cultivated plants in the tropics.....	about 34,500 kg.

Equally variable figures are required to express the ratio between the total amount of CO₂ in the air and that used each year by photosynthesis. Sachs calculated that there are 2500×10^{12} kg. of CO₂ in the atmosphere. Since plant assimilation uses 54×10^{12} kg. per month, all the CO₂ would be used in 4 years. Timiriazeff assumes that all the CO₂ of the atmosphere would be used in even less time. According to Schröder,⁴ there are 2100×10^{12} kg. of CO₂ in the air and the annual consumption is 60×10^{12} kg.; hence the atmospheric CO₂ would nourish the plants for 35 years without returning any to the air. Reinau⁵ estimates the supply of atmospheric carbonic acid to be 1530×10^{12} kg. and the annual consumption to be 86.5×10^{12} kg.; hence the CO₂ in the air would last 18 years. This writer is correct in noting that the herbaceous plants

¹ It is assumed that every sunflower occupies an area of 1 m².

² Mayer, A. Landwirt. Versuchs-Sta. 40: 203. 1892.

³ Becquerel. La lumière. 1868 Timiriazeff. Agriculture and plant physiology. 1906 (Russian), Ebermayer. Sitzungsber. Bayer. Akad. 15: 303. 1885.

⁴ Schröder, H. Naturwissenschaften. 7: 976. 1919.

⁵ Reinau. Chem. Zeit. P. 43. 1919.

grow under better conditions than trees and shrubs because the CO_2 content of the air is greater near the earth than at higher levels. On account of the ecological conditions, the "specific energy" must be higher for herbs than for trees.

Although there is a great discrepancy in these calculations, all the data gives the same results—the atmospheric carbon dioxide would be exhausted in a very short time. But since this does not occur there must be a corresponding production of CO_2 in nature. Carbon actually goes in the following cycle: assimilated by green plants in the light, it arrives in their bodies as organic compounds and passes to the animals in the same condition. Most of the carbon returns to the air as carbon dioxide of respiration, because every organism uses much more organic substance for its activities than for its structure. For example, a man weighing 70 kilograms gives off 100 kg. of carbon *every year* in the form of respiratory carbon dioxide. According to Timiriazeff,¹ "there is sufficient food for life because the air contains enough oxygen for its respiration, since nutrition depends on the removal of oxygen from carbon dioxide: every atom of carbon in food corresponds to an amount of oxygen in the air which is just enough to change this atom of carbon back to CO_2 ." By his respiration, according to approximate estimates, man annually excretes 438×10^9 kg. of CO_2 , which is about $\frac{1}{5000}$ th of the supply in the atmosphere. The respiration of green plants is about one-tenth of their photosynthesis in bright sunlight.² However, if it is taken into consideration that respiration continues night and day, we can agree with Sachs that the rate of respiration is about a fifth of that of assimilation. Considerably smaller amounts are released by the respiration of animals. The carbon which does not return to the air by the process of respiration enters the soil with the waste products of animals or in the dead bodies of plants and animals. All these substances are decomposed by the various processes of fermentation (Chap. VIII), the products of which are simpler organic substances. They are oxidized by various kinds of micro-organisms so that almost all the carbon of the atmosphere taken in by plants is finally returned. Peat and other plant residues which remain in the soil are the exceptions. New amounts of CO_2 thrown into the air by volcanic eruptions are introduced into the cycle and this replaces the carbon tied up in the soil. Man's activities also act in the same sense. He causes to pass into the air by his industrial operations about $2\frac{1}{2}$ times as much carbon as he gives off by his respiration. All steamships, railroads, factories, etc. change so much coal to CO_2 that the total volume of CO_2 released must have a geochemical significance. It has been calculated that the Krupp works at Essen alone previous to the World War transferred to the atmosphere annually not less than 900 million kilograms of carbon in the form of CO_2 .³ This amount of CO_2 is enough to feed a hundred thousand hectares of forest for several years.

¹ Timiriazeff, K. *loc. cit.*, p. 342.

² Boussingault, J. B. *Agronomie, chim. agr. et physiol.* 4: 267. 1860; Willstätter und Stoll. *Untersuchungen über Assimilation der Kohlensäure*, p. 326. 1918; Kostytschew, S. *Ber. bot. Ges.* 39: 319. 1921; and others.

³ Noll, F. *Bonner Lehrbuch.* 1st ed., p. 166. 1894.

The oceans are the regulators of the percentage of carbon dioxide in the air because a rise in the partial pressure of this gas in the atmosphere also increases the percentage in the water of the ocean. Consequently the analyses of sea water might give accurate indications as to changes in the CO_2 content in nature. Here we finally end in questionable estimates. There is the view that the CO_2 content of the sea water has increased on the average but other workers believe that it has remained constant because the fluctuations during the last 100 years have kept within the limits of the errors of analysis.

On the basis of numerous analyses of air, Lundegårdh¹ concludes that the CO_2 content of the atmosphere fluctuates widely—a fact which is entirely confirmed by the equally numerous analyses made by the author of this book and his co-workers. Accordingly there can be no question of a supply of CO_2 in the atmosphere; it is only a matter of a dynamic equilibrium between the production and the assimilation of CO_2 . That the latter process far outweighs the former is to be seen from the fact that the entire supply of oxygen of the atmosphere really comes through the work of chlorophyll bearers.² We actually know of no other natural process which liberates oxygen and we cannot imagine any other such process. On the other hand, it is just as unlikely that free oxygen would have been necessary for the origin of the earth. Hence there should be contained in the earth the quantities of carbon equivalent to the oxygen of the atmosphere.

According to Lundegårdh (*loc. cit.*), of all the present sources of CO_2 only the respiration of the soil is a process of the same order of magnitude as the assimilation of CO_2 by green plants. In the soil there is a huge amount of protoplasm and a large surface. According to Lundegårdh, the respiration of roots yields about one-fourth to one-third of the entire CO_2 production of the soil. By far the largest amount of CO_2 is therefore produced by the micro-organisms of the soil. Thus most of the mobile carbon passes from the soil to the lower strata of the atmosphere and then returns to the soil in the bodies of the green plants.¹

19. CARBON AND NITROGEN FERTILIZERS

We have convinced ourselves that plants under natural conditions do not grow in an optimum concentration of CO_2 and the nitrogen situation is worse. Soils are seldom found which do not need nitrogen fertilizers. It was shown above that there are only small reserves of nitrogen in the soil. Plants often exist on a patch of soil only at the expense of the nitrogen which enters it through the decomposition of plants which have grown there before. Small, inevitable losses of nitrogen are balanced by the rain which contains a certain amount of ammonia and oxidised nitrogen. The microorganisms which live in the soil also play a prominent rôle in the fixation of nitrogen, since they have the power to assimilate the nitrogen of the atmosphere (see the next chapter). This picture of a fairly well balanced existence changes for the worse when the land is used for agriculture. Then the whole crop of a growing season with all

¹ Lundegårdh, H. Der Kreislauf der Kohlensäure in der Natur. 1924.

² Arrhenius, S. Das Werden der Welten. 1906; Phipson, T. L. Chem. News. 67: 135. 1893; 68: 45. 1894.

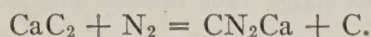
¹ The idea that this potential humus and therefore all manures and organic fertilizers work primarily because they add CO_2 to the soil as they are decomposed, has led to the writing of an entire book on the subject by E. Reinau (Praktische Kohlensäuredüngung. 1927). The four chapters deal with the principles, history, practical technique and the science of CO_2 fertilizers.—Ed.

the nitrogen contained in it is drawn away from the field. Hence the soil soon becomes poor in nitrogen and useless for plant growth if it is used for cultivation without adding nitrogen fertilizers each year. It was pointed out on the foregoing pages that plants suffering from lack of nitrogen assimilate CO_2 weakly and thus synthesize small amounts of organic substances. The nitrogen fertilizer must be imported because there are always some losses from farms. Cow manure is the only source of domestic fertilizer but it is evident that the use of this fertilizer returns to the soil only a small part of the nitrogen that was once removed. That held in the form of various foods is missing and all that assimilated by the cow and changed into animal proteins is not replaced in the soil. More will be said later about the importance of manure as a *carbon fertilizer*.

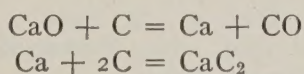
From this it is evident that every more or less well managed farm must receive the necessary amounts of nitrogenous fertilizer. Chile saltpeter has long been a useful fertilizer, which is found as deposits in the arid regions of Chile and southern Peru,¹ and together with guano (bird droppings) which is an even better fertilizer, formed the chief export from these states for many years. Saltpeter has the advantage that it can be used by all plants on all soils. The beds of saltpeter are now nearly exhausted and the discovery of a satisfactory substitute was the pressing need of this century. The first fertilizer to be used much in its place with outstanding success is ammonium sulphate, a by-product in the manufacture of illuminating gas. The conditions for using ammoniacal fertilizers were described above (p. 117) but ammonia can easily be oxidized to nitric acid. Still ammonia comes largely from coal and this source cannot continue to meet the growing needs. With reference to the nitrogenous by-products of slaughter-houses and the various industries, they are to be regarded as secondary fertilizers because their total amount is so small. Consequently there has been a considerable increase in the cost of nitrogenous fertilizers in recent years.

The only inexhaustible source of nitrogen is the air. To use it was a problem for modern technique. It requires a high consumption of energy to change molecular nitrogen to the compounds of nitrogen. In regions where waste power can be used to produce high voltage electricity, *i.e.*, where there are water falls and swift-flowing streams, it is profitable to oxidize the nitrogen with atmospheric oxygen in a Volta arc with metal electrodes. Nitric acid is the product of this oxidation. A simpler method of forming saltpeter is to heap up manure, peat and other nitrogenous by-products, putting in layers of brush to aerate it, and to water it with urine, manure drainage and similar liquids. The nitrogenous substances are mineralized by bacterial action and ammonia is split off and converted to nitric acid by the nitrifying bacteria (see the next chapter). The danger in this method of producing saltpeter is the possibility of denitrification (see next chapter) unexpectedly entering and causing the loss of the entire supply of nitrates. For this reason it is perhaps better to encourage nitrification in the soil and then to fertilize with substances containing nitrogen. Nitrification is aided by loosening the soil to allow the entry of air, by adding lime and by some other things.

Another fertilizer prepared at the expense of atmospheric nitrogen is the so-called "lime-nitrogen" or the calcium derivative of cyanamide $\text{CN}(\text{NH}_2)$.² It is made from nitrogen and calcium carbide at a temperature of $1100-1200^\circ$.³ If 10% of anhydrous calcium chloride is added to the carbide, a temperature of $700-800^\circ$ is sufficient.⁴



The product is used to fertilize the soil without removing the impurity of carbon. Calcium carbide is prepared by melting anhydrous calcium oxide with coke in an electric oven at $2500-3500^\circ$.⁵



¹ Thiercelin. Ann. de chim. et de physique (4) 13: 160. 1868; Plagemann. Der Chilesalpeter. 1905.

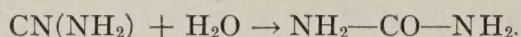
² Both the hydrogen atoms of cyanamide can be replaced by metals.

³ Frank, A. and N. Caro. German Pat. 108971; Idem. Chem. Centralbl. 1: 1120. 1900.

⁴ Polzeniusz. German Pat. 163320; Chem. Zeit. 31: 958. 1907.

⁵ Moissan. Compt. rend. 118: 501. 1894; Traité de chimie minérale. 3: 690. 1904.

In the soil the "lime nitrogen" changes to cyanamide which readily becomes urea by the addition of water:¹



Common microorganisms in the soil bring about this change.² Then they remove ammonia from the urea so that the effect is like that of ammoniacal fertilizers.³ Of itself, cyanamide is toxic to all plants and if it were not for the opportune transformation in the soil it might produce unfavorable results.

The hard years of the World War produced important results in the field of the fertilizer problem. In connection with the food crisis it was necessary to grow as many crop plants as possible and to find new ways to improve the utilization of solar energy by plants. It was found that the work of green plants on well fertilized soil and with plenty of nitrogen is limited by the low content of carbon dioxide in the air. This led to the bold idea of enriching the air with carbon dioxide for intensive farming. The first experiments in this direction were done some time ago⁴ but they gave unfavorable results, probably because there were impurities in the carbon dioxide used.⁵ In some sections, experiments in fertilizing plants by increasing the CO₂ content of the air are being tried on a large scale. Particular interest attaches to the use of purified gases from blast furnaces.⁶ These have given astonishing results. The dry weight of the crop increases about 400% in comparison with normal crops and no such results can be had with soil fertilizers. Of course, the amount of carbohydrate in the crop increases while the percentage of protein remains normal. In the opinion of some of the specialists in the matter, the fertilization of plants through the air has a bright future while others are reserving judgment.⁷ The supporters of air fertilizers point to the long recognized fact that manures are indispensable even when there is an abundant supply of mineral nutrients. They explain it by saying that manure furnishes carbon as well as nitrogen because the decomposition of the manure by soil microorganisms provides a large quantity of carbonic acid which is used by the cultivated plants.

Greater advances have been made in the field of nitrogen fertilizers. In 1913 Haber⁸ solved the problem of synthesizing ammonia from its elements. The difficulty of this problem was the need of providing the temperature of a red glow to make the nitrogen unite with hydrogen, but even then so little ammonia was obtained that the output was not worth mentioning. The lower the temperature the more complete is the synthesis of ammonia but the harder it is to start the reaction. This difficulty was overcome by having the elements combine in a current of gas with the temperature not over 700° but under pressure and in the presence of suitable metallic catalysts.⁹ The yield of ammonia depends on the successful choice of catalysts. The manufacture of ammonia was highly organized in Germany during the World War. All of the nitric acid required for war purposes was obtained by the oxidation of ammonia prepared by the Haber process so that Germany is no longer dependent of Chile saltpeter.

This method of fixing atmospheric nitrogen is so cheap that it is displacing all other methods of preparing nitric acid from the air. The manufacture of ammonia from its elements is admitted by all to be one of the greatest technical achievements of all time. Moreover, it is one of the brilliant victories of mankind over hostile nature. The Damocles sword of the

¹ Ulpiani. *Gaz. Chim. Ital.* 38 (II): 399. 1908.

² Aeby. *Jour. Agr. Sci.* 1: 358. 1905.

³ Mazé, Vila et Lemoigne. *Compt. rend.* 169: 921. 1919.

⁴ Brown, H. T. and F. Escombe. *Proc. Roy. Soc. London (B)* 70: 402. 1902; Farmer, B. and S. E. Schandler. *Ibid.* 70: 413. 1902.

⁵ Lundegårdh, H. *Zeitschr. f. angew. Bot.* 4: 120. 1922.

⁶ Riedel, F. *Stahl und Eisen.* 39: 1497. 1919; Fischer, H. *Gartenflora.* 68: 165. 1919; Idem. *Naturwiss. Wochenschr.* 19: 177. 1920; and others.

⁷ On this point cf. Mitscherlich, E. A. *Zeitschr. f. Pflanzenernährung.* 2: 24. 1923; Spargatis, P. *Bot. Arch.* 4: 381. 1923.

⁸ Haber, F. und van Oordt. *Zeitschr. f. anorg. Chem.* 44: 342. 1905; Haber, F. und R. le Rossignol. *Zeitschr. f. Elektrochem.* 19: 53. 1913.

⁹ New patents announce considerably more of a reduction in temperature.

exhaustion of nature's stores of fixed nitrogen has ceased to hang over our heads and we can now view calmly the extermination of the deposits of Chile saltpeter.

For agricultural crops on unexhausted soils and soils not requiring excessive amounts of mineral fertilizers, there is also the following possibility of providing nitrogen. It is known that the supply of nitrogen in such soils can be assured for plant nutrition for several decades if the nitrogen passes gradually into the form in which it can be assimilated. This can be brought about by a suitable fertilization, change of pH and aeration of the soil.¹

By suddenly killing a large part of the soil bacteria by poisoning or drying the soil, the nitrogenous constituents would be placed at the disposal of the seed-plants. Yet such a sudden mineralization of the organic nitrogen of the soil, which usually increases the harvest in the first year, is hardly to be recommended for continual practice because, in the first place, many useful microorganisms are lost and replaced by undesirable forms, and secondly, there is an excess of nitrogen which can be assimilated. The seed-plants are unable to take in all this nitrogen. Part of it goes for material for denitrification (p. 160) and part as food for new-coming bacteria. There can thus be a considerable loss of the nitrogen supply of the soil.

¹ See the papers in *Bur. f. landwirt. Mikrobiologie in Leningrad*, vols. 1-3. 1926-1928.

CHAPTER III

CHEMOSYNTHESIS AND THE ASSIMILATION OF MOLECULAR NITROGEN

1. THE GENERAL IDEA OF CHEMOSYNTHESIS

The synthesis of organic substances from mineral substances is not effected by green plants alone. Some bacteria which have no pigments sensitive to light, feed on mineral substances just as exclusively as the green plants but to perform their syntheses they use not the energy from the sun but that of exothermal reactions which yield an abundance of heat. Examples of such reactions are the oxidation of hydrogen to water, of hydrogen sulphide to sulphur and sulphuric acid, etc.

These remarkable phenomena are the result of specific adaptations of various bacteria to certain chemical operations which are not otherwise peculiar and which go on to completion like all processes which release energy. Analogous specific adaptations of some bacteria to certain chemical reactions are the fermentations described in Chap. VIII, which are also processes which release energy. It must be noted that chemosynthesis has not the cosmic importance which attaches to photosynthesis, by which organic substances are formed at the expense of the free energy of sunlight and radiant energy is stored up. Chemosynthesis transfers to a certain substance the potential energy stored in another substance. From one kind of energy it makes another kind which is required by the organism concerned but there is no storage of energy. The total store of energy at high tension, which serves as a measure of the development of life on our planet, is not increased. To use an analogy, photosynthesis may be likened to the making of money and chemosynthesis to an exchange of money which usually involves a small loss.

The enormous biological significance of chemosynthesis manifests itself in an entirely different realm. The reactions which characterize chemosynthesis are responsible for the cycle of some physiologically important substances which are used in the bodies of plants and animals. Chemosynthesis changes these substances to a form in which they can be used by plants. These conditions will be discussed later.

The existence of chemosynthesis shows that light is not always necessary for the synthesis of biologically important organic substances.

2. NITRIFICATION

Practically all the nitric acid in the soil is of biological origin. It is formed by the oxidation of ammonia which itself appears at the expense of the nitrogen of proteins and their cleavage products during the decay of the dead bodies of

animals and plants (p. 117). Nitrification is the oxidation of soil ammonia to nitric acid. It has long been thought that nitrification must be a biological process¹ because it is stopped by the action of various anaesthetics and poisonous substances, such as chloroform. The epochal investigations of Winogradsky² explained the nature of these peculiar processes and disclosed many new and unexpected relationships.

The chief difficulty in the isolation and culture of nitrifying organisms, which has bothered a series of investigators, is that the usual bacteriological methods do not give satisfactory results. The plating method is used to isolate microorganisms. The medium to be studied is distributed in sterilized (*i.e.*, free from living forms) water, a few drops are transferred to liquified, sterile gelatine or agar and the latter is then poured into a suitable number of flat, sterilized petri dishes. The cooling of the jelly-like substrate holds each microorganism in a fixed position and the individuals which grow from it form a colony of a single species. These colonies often have a characteristic appearance. Organisms from a single colony can easily be transferred to separate dishes and pure cultures thus obtained. A pure culture contains but one species of microorganisms.

Since this general method can never be used to isolate the nitrifying organisms, Winogradsky introduced the so-called "elective culture" method of isolating these forms. An elective culture is a growth of all the microorganisms of the source to be investigated, under artificial conditions which aid the isolated forms in their struggle for existence. These multiply fast and suppress the growth of other organisms. As a result, they accumulate in such quantities that it facilitates subsequent isolation of pure cultures. In the case in which we are interested at this point, one might expect that the nitrifying forms would depend less on organic substances than other microorganisms, because the working energy, which usually comes from respiration, *i.e.*, the combustion of organic substances, in the case of nitrification is provided by the oxidation of ammonia, an inorganic substance. For this reason Winogradsky used potassium tartrate as the only organic substance in elective cultures. When he found that these conditions were inadequate, Winogradsky resolved to put no organic compounds in his cultures. The nutrient solution contained only 1 g. of ammonium sulphate and 1 g. of potassium phosphate per liter. In addition there was a precipitate of 5-10 g. of magnesium carbonate on the bottom of the vessel. The magnesium was provided in the form of a carbonate in order to neutralize the nitric acid formed in the liquid. This method was entirely successful; there was just as much nitrification in the liquid as under natural conditions. Of course, the majority of the microorganisms of the soil did not even develop in the absence of organic substances. The pellicle of mold which formed on the surface of the solution contained only 5 kinds of bacteria. A new difficulty

¹ Schloesing, Th. et A. Muntz. Compt. rend. 84: 301. 1877; 85: 1018. 1877; 86: 892. 1878; 89: 891 and 1074. 1879; Warington. Centralbl. f. Bakt. 6: 498. 1888; Frankland, G. and P. Zeitschr. f. Hyg. und Infektionskrankheit (II) 2: 415. 1896.

² Winogradsky, S. Ann. Inst. Pasteur. 4: 213, 257 and 760. 1890; 5: 92 and 577. 1891; Idem. Centralbl. f. Bakt. (II) 2: 415. 1896.

of this delicate investigation appeared in that not one of the 5 bacteria was able to bring about nitrification. The active agents were finally found on the bottom of the vessel, in the precipitate of magnesium carbonate, although it was more sensible to look for them at the surface in direct contact with the atmospheric oxygen required to oxidize the ammonia.

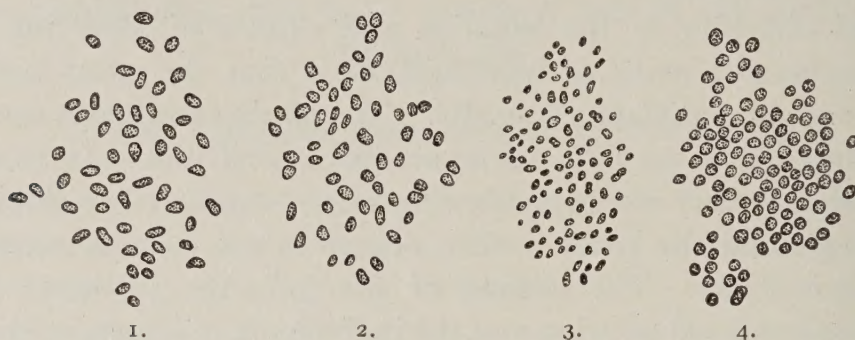


FIG. 20.—Nitrite formers from different soils: 1. from Zurich; 2. from Jeanvillier near Paris; 3. from Kasan; 4. from Petersburg. Magnified 1000 $\times$. (After Winogradsky and Omeliansky.)

Great technical difficulties attended the isolation of the nitrifying forms in pure culture, because a solid substrate had to be found which would be free from organic impurities. Neither gelatine nor agar could be used. Finally Winogradsky succeeded in isolating pure cultures on silicic acid jelly. Now we

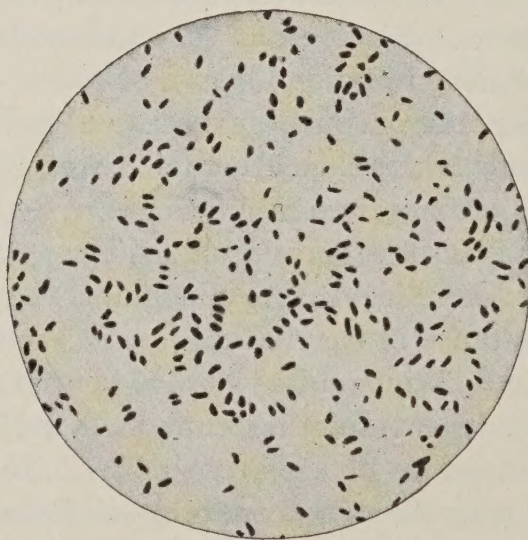


FIG. 21.—*Nitrobacter*. Magnified 1000 $\times$. (After Winogradsky and Omeliansky.)

are using for this purpose plates of a mixture of gypsum and magnesia on solid paper plates.¹

Experiments with pure cultures showed that ammonia was often oxidized only as far as nitrous acid, even under the most favorable conditions. More

¹ Omeliansky, W. *Centralbl. f. Bakt.* (2) 5: 537. 1899; 8: 785. 1902; cf. also Perotti, R. *Atti Reale Accad. dei Lincei. Rend.* (5) 14 (II): 228. 1905; Duggeli. *Naturwiss. Wochenschr.* 14: 305. 1915.

careful study showed that there are two kinds of nitrifying organisms:¹ the one oxidizes ammonia as far as nitrites while the other oxidizes the nitrites to nitrates but is unable to work on ammonia which actually poisons it severely.

The nitrite organisms in Europe and America are not identical: in the Old World they are oval with numerous long flagellae (*Nitrosomonas*, Fig. 20), but in America the nitrite-forming bacteria resemble a small coccus (*Nitrosococcus*). The nitrate former (*Nitrobacter*) is a short rod (Fig. 21). This organism is one of the smallest bacteria.

The best organic foods are very toxic to both nitrite and nitrate formers. Sugar is just as much of a poison for them as phenol is for other bacteria, and ammonia is as injurious to the nitrate formers as sublimate is for other organisms. The following table shows the minimum amounts of organic substances which check the development of the nitrifying bacteria.²

Compounds	Nitrite formers		Nitrate formers	
	Growth retarded	Growth stopped	Growth retarded	Growth stopped
Grape sugar.....	0.025%	0.05%	0.05%	0.2%
Peptone.....	0.025%	0.2%	0.8%	1.25%
Asparagine.....	0.025%	0.3%	0.05%	0.5%
Glycerine.....	<0.2%		0.05%	<1.0%
Urea.....	<0.2%		0.5%	<1.0%
Sodium acetate.....	0.5%	<1.5%	1.5%	3.0%
Ammonia.....			0.0005%	0.015%

This remarkable negative behavior toward organic substances, especially toward good nutritive materials, indicates chemosynthesis without a doubt. It was also found that these peculiar bacteria, like the green plants, are nourished by the CO₂ of the atmosphere³ and the inorganic nitrogen compounds. Of special interest is the unparalleled behavior of these organisms toward sugar, the universal foodstuff, which is found in every living cell. It should also be noted that the nitrifying bacteria, for unknown reasons, can thrive in natural soils which contain considerable amounts of the most varied kinds of organic substances.⁴

It was most interesting to show whether the nitrifying bacteria had the ordinary oxygen respiration at the expense of organic compounds, in addition to the oxidation of ammonia and nitrites. Meyerhof concludes that the

¹ Winogradsky, S. Arch. Inst. f. Exp. Med. 1: 87. 1892 (Russian).

² Winogradsky, S. und Omeliansky. Centralbl. f. Bakt. (II) 5: 338, 377 and 429. 1899. Confirmed by Meyerhof, O. Pflügers Arch. f. ges. Physiol. 165: 229. 1916; 166: 240. 1917.

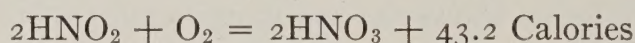
³ Godlewski, E. Bull. L'Acad. Sci. Cracovie. 1895; Idem. Centralbl. f. Bakt. (II) 2: 458. 1896.

⁴ Karpinski, A. et Br. Niklewski. Bull. l'Acad. Sci. Cracovie, p. 596. 1907; Coleman, L. C. Centralbl. f. Bakt. (II) 20: 401. 1908; Mazé. Compt. rend. 152: 1625. 1911; Greaves and Carter. Jour. Agr. Res. 6: 889. 1916; Wright. Centralbl. f. Bakt. (II) 46: 74. 1916; Barthel. Ibid. 49: 382. 1919.

nitrate formers lack a normal respiration.¹ Comparison of the quantities of oxidized nitrite and of oxygen used shows that there is no other oxidation.

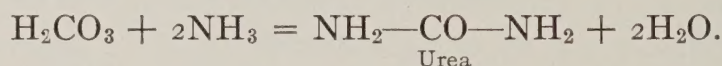
O ₂ Consumed	O ₂ Required for Nitrite Oxidation
0.270 cc.	0.273 cc.
0.392 cc.	0.396 cc.
0.277 cc.	0.290 cc.
0.293 cc.	0.295 cc.
0.244 cc.	0.240 cc.

Hence the total gas exchange of nitrate formers is said to be expressed by the equation:

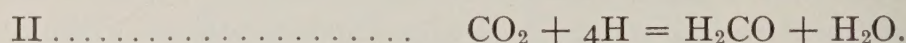
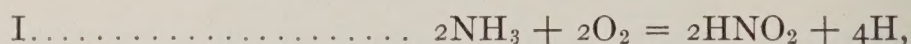


It is to be observed, however, that the oxygen respiration in the foregoing experiments must remain within the limits of the experimental error.

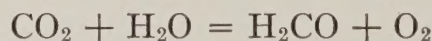
Winogradsky² proposed a scheme of CO₂ assimilation by nitrifying bacteria according to which no oxygen is liberated by the chemosynthesis and no carbohydrate is formed:



Urea (see Chap. VI) can indeed be used as material for protein synthesis but only in the presence of sugar, from which the carbon framework of amino acids is built. The other theories which have been suggested by different writers to explain chemosynthesis are not at all alike. According to one, the nitrifying bacteria form formaldehyde and even sugar but without giving off oxygen:³



According to others, chemosynthesis is entirely analogous to photosynthesis:⁴



The first theory is fundamentally improbable because each atom of carbon assimilated by the nitrate formers actually corresponds not to 2 but to about 35 molecules of oxidized ammonia.

At first glance it might appear that the question of liberation of oxygen by the chemosynthesis of nitrifying bacteria could be solved by direct gas analysis. Actually such analyses meet with great technical difficulties because the simultaneous oxidation of ammonia involves a large absorption of oxygen. If oxygen is released in the assimilation of CO₂, the amount of it is small in comparison to that absorbed from the air at the same time. For the nitrite formers,

¹ Meyerhof, O. Pflügers Arch. f. ges. Physiol. 164: 353. 1916.

² Winogradsky, S. Compt. rend. 110: 1016. 1890.

³ Loew, O. Bot. Centralbl. 46: 222. 1891.

⁴ Hüppe. Naturwiss. Einführung in die Bakteriologie, p. 66. 1896.

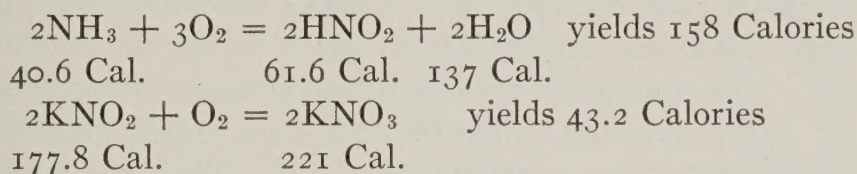
Winogradsky found the following ratios between the carbon assimilated and the nitrogen oxidized:

Nitrogen oxidized.....	722.0	506.1	928.3	815.4
Carbon assimilated.....	19.7	15.2	26.4	22.4
Ratio of <i>N</i> to <i>C</i>	36.6	33.3	35.2	36.4

Another fairly constant ratio of *N* to *C* is obtained by Meyerhof (*loc. cit.*) for *Nitrobacter* (a nitrate former):

Nitrogen oxidized.....	475	466	385	378
Carbon assimilated.....	3.52	3.55	2.63	2.95
Ratio of <i>N</i> to <i>C</i>	135	131	146	128

The nitrite formers thus assimilate one atom of carbon to 35 atoms of nitrogen, and the nitrate formers one to 135 molecules of nitrite. The quantitative difference corresponds to the inequality of the heat production in the oxidation of ammonia and nitrite:



In connection with the table on p. 152, which gives the ratio of oxygen absorbed to nitrite oxidized, if it is taken into consideration that $\frac{N}{C} = 135$, one can convince himself that if oxygen is formed during the assimilation of CO_2 , it cannot exceed the limits of error of analysis (cf. also p. 152).

The nitrifying bacteria are an interesting and instructive example of the so-called bacterial partnership. Both are closely bound to each other. The nitrite builders profit by the removal of nitrite which would decrease their vital activities. This work is done by the nitrate formers which cannot live apart from the nitrite builders because only in their company do they receive nitrites and protection from the ammonia which injures the nitrate formers. But the successful life of this partnership is impossible without the simultaneous work of other microorganisms which break down organic compounds of nitrogen and thereby form NH_3 . These are the ordinary saprophytes—bacteria and fungi—which occur in every soil and belong to various species. They always live in the immediate vicinity of nitrifying bacteria and furnish the necessary ammonia. By extensive bacteriological studies of soils and streams, Winogradsky showed that the nitrifying bacteria which he had discovered are generally widespread and that the formation of nitric acid in soils, fresh water and sea water is due to the activities of these bacteria.¹ Nitrification takes place rapidly only in well aerated, non-acid soils. The deposits of saltpeter in Chile are undoubtedly the result of the activities of the bacteria described

¹ Cf. also Weiss, F. Centralbl. f. Bakt. 28: 434. 1910; Fremlin, H. S. Proc. Roy. Soc. London, Vol. 71. 1903; Stekens, F. L. and W. A. Withers. Centralbl. f. Bakt. 34: 187. 1912; Kellermann, K. F. and T. R. Robinson. Science. 30: 413. 1910.

by Winogradsky. Contrary to the views of earlier investigators, nitrification is possible in woodland and moor soils.¹ It has been proved that the small quantities of oxidized nitrogen which are formed in the atmosphere from the oxidation of molecular nitrogen by oxygen and ozone during electrical discharges, in comparison with the nitrates of biological origin, are too small to estimate.

Different investigators have repeatedly described other nitrifying organisms which are said to differ from those discovered by Winogradsky, but these findings have always been revoked. Recent evidence indicates that the power to oxidize ammonia is also possessed by one of the *Actinomycetes*,² i.e., organisms which are intermediate between bacteria and fungi, and by 4 new species of *Nitrobacter* which supposedly split cellulose and are not poisoned by NH_3 .³ As to how far these claims are correct and the organisms concerned are significant in the nitrogen cycle, remains uncertain.

The extent of nitrification under natural conditions⁴ is extraordinary. It has also been shown that during a wet summer the nitrifying bacteria in a fallow, well aerated soil can accumulate as much as 200 kg. of oxidized nitrogen per hectare. This amount is entirely sufficient for any intensive agricultural use of the soil. It is interesting to find that the nitrifying bacteria occur on the bare lime rocks above the zone of plant growth.⁵ They are pioneers of life everywhere that lime is present to neutralize the acids which they form. These organisms require nothing from the earth; the air provides all they need for life. From the precipitation they receive ammonia, water and nitrous acid (the latter is a result of electrical phenomena).^j A sufficient amount of mineral substances is obtained in solution from the lime and dolomite rocks through the resulting acids. We cannot help but think that the nitrifying organisms have all the characteristics required by the first life on our planet, since the latter naturally started without organic foodstuffs.^k It is interesting that the size of the nitrifying bacteria is that of the little particles which Arrhenius⁶ calculated might be borne through space by the pressure of light waves.

In conclusion it should be noted that the nitrifying bacteria are specific; they are able to oxidize ammonia and nitrites but not urea and amines.⁷ In the same way they are unable to use sulphurous and phosphorous acids⁸ although

¹ Migula, W. Centralbl. f. Bakt. (II) 6: 365. 1900; Vogel v. Falckenstein. Jour. f. Landwirtschaft. 62: 173. 1914. Nitrification also takes place in waste waters: Reid. Proc. Roy. Soc. London (B) 79: 58. 1907; Dienert. Compt. rend. 165: 1116. 1917; Nashsmith and McKay. Jour. Ind. Eng. Chem. 10: 339. 1918; Rundick. Ibid. 10: 400. 1918. Nitrification in moor soils: Arndt, Th. Centralbl. f. Bakt. 49: 1. 1919.

² Joshi. Mem. Dept. India Bact. 1: 85. 1915.

³ Sack, J. Centralbl. f. Bakt. (II) 62: 15. 1924.

⁴ Papers of the Bur. f. Landwirt. Mikrobiologie in Leningrad. Vols. 1-4. 1926-1929.

⁵ Muntz. Compt. rend. 111: 1370. 1890.

^j Sulphur is also a component of rainwater, especially in regions where coal is burned. Cf. Eaton, S. V. and J. H. Eaton. Plant Physiology. 1: 77-87. 1926.—Ed.

^k This hypothesis is well explained by H. F. Osborn in his book entitled The Origin and Evolution of Life. A part of the evidence used in support of this hypothesis consists of the fossil bacteria reported by Wolcott.—Ed.

⁶ Arrhenius, S. Das Werden der Welten. 1907.

⁷ Omeliansky, W. Centralbl. f. Bakt. (II) 5: 481. 1899.

⁸ Omeliansky, W. Ibid. 9: 63. 1902.

the oxidation of these acids is, from their nature, strictly analogous to the mechanism of oxidation of nitrous acid. The latter can also be used only in the ionized condition.¹

3. OXIDATION OF HYDROGEN SULPHIDE

Almost no higher or lower plants grow in the sulphur springs which contain dissolved hydrogen sulphide, because the latter is toxic to them even in dilute concentrations. A very peculiar vegetation appears in water containing much hydrogen sulphide. A particularly striking form is the colorless bacterium *Beggiatoa* (Fig. 22) which consists of filaments filled with grains of sulphur. *Beggiatoa* is also found in soils containing sulphur and for this reason it was long thought that it reduces sulphuric acid to hydrogen sulphide by way of molecular sulphur, and this was one of the reasons for the formation of this gas in sulphur springs. This hypothesis proved to be incorrect. Sulphate is important for *Beggiatoa* only in so far as it represents material for the formation of hydrogen sulphide by various other biological processes. *Beggiatoa* itself requires only the hydrogen sulphide. Winogradsky² explained the remarkable biochemical characteristics of *Beggiatoa*.

The work of this investigator showed that *Beggiatoa* oxidizes hydrogen sulphide to sulphur for the energy of the process, just as the nitrifying bacteria are able to synthesize organic substances by the use of such energy. In *Beggiatoa* the characteristic features of a chemosynthesizing organism are not so evident

as in the nitrifying bacteria; still it does effect chemosynthesis because Winogradsky was able to grow it for a long time in a natural mineral water, rich in hydrogen sulphide, and which contained not more than 0.0005% organic substances in the form of salts of formic and propionic acids. All experiments designed to increase the content but particularly the food value of organic substances in culture fluids at once checked the oxidation of H_2S and resulted in the death of the bacterial filaments.

If the behavior of the bacteria in water containing H_2S is observed under the microscope, it is easy to see that they are in constant motion. As they approach the edge of the cover glass they obtain a supply of atmospheric oxygen and then return to oxidize H_2S at the center of the preparation. In a short

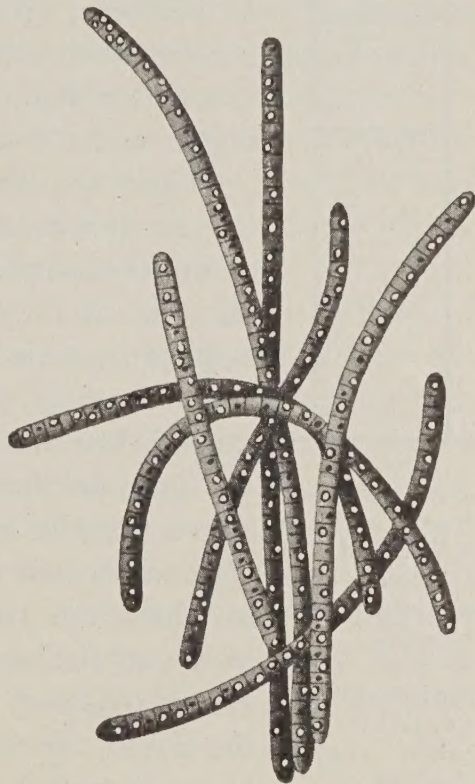


FIG. 22.—*Beggiatoa*. Magnified 1000 X. (After Omeliansky.)

¹ Meyerhof, O. Pflügers Arch. f. ges. Physiol. 164: 353. 1916.

² Winogradsky, S. Bot. Zeit. 45: 489. 1887; Idem. Ann. Inst. Pasteur. 3: 49. 1889; Idem. Beitr. z. Morph. und Physiol. d. Bakterien, Vol. 1. 1888. The latest monographs are: Dügge, M. Neujahrsbl. d. Naturforscherges. zu Zurich. Vol. 121. 1919.

time the filaments are filled with sulphur which probably occurs in the liquid condition. In the presence of calcium carbonate, the sulphur is oxidized, forming sulphuric acid which then becomes gypsum [CaSO_4]. Pure cultures of *Beggiatoa* can be grown in solutions free from organic substances.¹ The cultures oxidize hydrogen sulphide and assimilate atmospheric carbon dioxide for the synthesis of organic substances.² Unlike the nitrifying bacteria, *Beggiatoa* in pure cultures tolerates a rather large amount of organic substances.

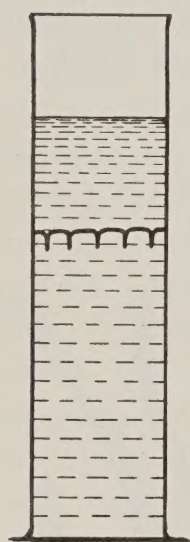


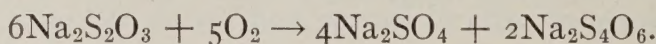
FIG. 23.—
Film of sulphur bacteria in a cylinder of H_2S water. The film sends down hanging projections which are called "bacterial fountains." (After Jegunoff.)

It is very interesting that there are enormous numbers of H_2S -oxidizing bacteria in the Black Sea.³ In the deep parts the water contains so much hydrogen sulphide that there are no deep-sea forms of life there. At a depth of about 200 meters,⁴ the Black Sea has a zone filled with sulphur bacteria. They are spiral in form and absorb the H_2S so fast that there is not a trace of this gas at the higher water levels.

These spirillae appear to differ from *Beggiatoa* in that they can live an anaerobic life. It is hard to account for the enormous amounts of sulphuric acid in the Black Sea. The activity of these bacteria can be reproduced on a small scale as follows. If various sulphur bacteria are grown in a glass cylinder filled with hydrogen sulphide water (Fig. 23), they form a movable film at some distance below the surface.

Analysis shows that there is almost no hydrogen sulphide above the film but that sulphuric acid accumulates there. The plate of bacteria forms hanging projections ("bacterial fountains") in which the bacteria are very motile. There they obtain a supply of H_2S and take it to the upper part of the film where the H_2S is oxidized. According to the hydrogen sulphide content of the liquid, the film forms at such a depth that the bacteria have access to both hydrogen sulphide and oxygen.

There are different species of colorless sulphur bacteria. They all have about the same characteristics and endure high concentrations of H_2S . Later there were found some peculiar sulphur bacteria which carry on another oxidation. They change thiosulphates into sulphates and salts of tetrathionic acid.



These organisms do not suffer from the presence of organic compounds but they cannot use them for respiration. Thus they too are chemosynthetic forms.⁵

¹ Dangeård. Compt. rend. 153: 963. 1911.

² Keil. Cohns Beiträge. 11: 335. 1912.

³ Jegunoff. Arch. f. biol. Wiss. 3: 297. 1895 (Russian); Idem. Centralbl. f. Bakt. (II) 2: 11, 411, 478 and 739. 1896.

⁴ According to recent studies by Knipowitsch, the zone of H_2S does not lie at the same level in all parts of the Black Sea. At some points there is no normally respiring form of life even at a depth of 25 meters and from there down to 2000 m. or more. Cf. Knipowitsch, N. Compt. rend. acad. sci. de Russie, p. 63. 1924; Russ. Hydrbiol. Zeitschr. 3: 199. 1924 (Russian).

⁵ Nathansohn. Mitt. Zool. Stat. zu Neapel. 15: 655. 1902; Beijerinck. Centralbl. f. Bakt. (II) 11: 593. 1904.

The purple sulphur bacteria occupy a special position.¹ They differ from all the other sulphur bacteria, which tolerate only low concentrations of hydrogen sulphide, in that they thrive in water that is saturated with H_2S . This property can only be explained by the liberation of O_2 into the water by the bacteria themselves for the oxidation of the H_2S so that their protoplasm is not injured by this gas. The view has been expressed that purple bacteria always live in symbiosis with green algae and use the oxygen released by the latter in the light.² Another hypothesis states that the purple bacteria themselves give off oxygen in the light and are thus capable of true photosynthesis, like the green plants.³

All observations of purple bacteria have actually proved the favorable effect of light on their growth, in that light injures the colorless bacteria including *Beggiatoa*. The purple bacteria contain a red pigment—bacteriopurpurin⁴—which absorbs the ultra-red rays. Engelmann declares that these rays provide the purple bacteria with energy for photosynthesis. In addition, these bacteria contain a green pigment, bacteriochlorin, which differs from chlorophyll by the absence of the most important absorption band between *B* and *C*. A third pigment has been described as bacterioerythrin.⁵ Further investigations of the purple bacteria have for some time thrown the foregoing questions into confusion. Their photosynthetic activity has been questioned. Thus Molisch claimed⁶ that the purple bacteria, unlike the colorless sulphur bacteria, cannot live without organic foods. If this were true they would not need to use hydrogen sulphide as a source of energy. Recently convincing evidence has been furnished that there are purple bacteria which do not absolutely require organic nutrient. This brings Engelmann's view again to the foreground.⁷ To obtain more reliable results it will be necessary to work with pure cultures which have not been used for these forms. In addition, bacteria have been described recently as oxidizers of molecular sulphur.⁸

4. OXIDATION OF FERROUS AND MANGANOUS SALTS

Winogradsky⁹ described peculiar iron bacteria which oxidize ferrous to ferric salts and utilize the energy of these exothermal processes for chemosynthesis. The ferric salts are held for a time in the body of the organism but the iron is finally precipitated as iron hydroxide. The extent of the activity of these iron bacteria (species of *Leptothrix*, *Crenothrix*, *Spirophyllum*, etc.) is

¹ Ray-Lankester. Quart. Jour. Microscop. Sci. Vol. 13. 1873; Vol. 16. 1876.

² Winogradsky. Beitr. Morph. u. Physiol. d. Bakterien. 1: 51. 1888; [The presence of purple bacteria as symbionts in lichens was announced by Uphof (Amer. Jour. Bot. 12: 97-103. 1925; Science. 61: 67. 1925) but this has been contradicted by Suessenguth (Ber. bot. Ges. 44: 573-578. 1926) who claims that the color observed by Uphof was due to the presence of chiodectonic acid.]

³ Engelmann. Bot. Zeit. 46: 693. 1888; Idem. Pflügers Arch. f. ges. Physiol. 42: 183. 1888.

⁴ Ray-Lankester. loc. cit.; Nadson, G. Mitt. d. bot. Garten in Petersburg. 3: 102. 1903 (Russian); Molisch, H. Die Purpurbakterien. 1907.

⁵ Arzichowsky, W. Mitt. d. bot. Garten in Petersburg. 4: 1. 1904 (Russian); Lubimenko, W. Jour. Russ. Bot. Ges. 6: 107. 1923.

⁶ Molisch, H. loc. cit.

⁷ Skene, M. New. Phyt. 13: 1. 1914.

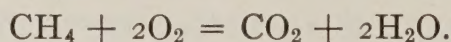
⁸ Waksman, S. A. Jour. Bact. 7: 231, 239, 602 and 609. 1922; Waksman and R. E. Starkey. Jour. Gen. Physiol. 5: 285. 1923.

⁹ Winogradsky, S. Bot. Zeit. 46: 261. 1888.

seen in that they are thought to have formed the deposits of so-called bog iron ore. By multiplying in water pipes the iron bacteria do great damage because they often stop up the pipes with iron hydroxide. Some bacteria which live in the presence of iron salts and were once thought to effect chemosynthesis, were later found to be ordinary saprophytes.¹ The existence of chemosynthesis was established in the others.² Iron bacteria often show a tendency to enter into symbiosis with the green algae, perhaps because they thereby obtain plenty of oxygen.³ Moreover it has been said that certain molds, such as *Citromyces siderophylus*, also store iron.⁴ Some iron bacteria oxidize manganous salts as well; examples are *Bacillus manganicus*, which stores manganic oxide⁵ and *Ferribacterium calceum*,⁶ which also stores lime.

5. OXIDATION OF HYDROGEN, METHANE, CARBON MONOXIDE, CHARCOAL, ETC.

In the peats and soils without oxygen there is an enormous decomposition of nitrogen-free organic substances which involves the liberation of hydrogen and methane CH₄ (see Chap. VIII). As the result of these processes, the hydrogen content of the atmosphere is scarcely less than the CO₂ content. Bacteria are now known which oxidize hydrogen to water. Among them are *Bacillus oligocarbophilus*, *B. pycnoticus*, *B. pantotrophus*, etc.⁷ Ruhland and Grohmann say that there are many of these species. Methane is oxidized to carbon dioxide and water by *Bac. methanicus*, *B. hexacarbovorum* and others:⁸



Hydrogen peroxide may be an intermediate product in the oxidation of hydrogen. A large amount of energy is obtained by the hydrogen bacteria through the combustion of hydrogen and they use it for chemosynthesis. They assimilate CO₂ of the atmosphere and liberate oxygen but only in the presence of iron salts.⁹ Unlike the nitrifying bacteria, they can live on organic substances and thus live in the absence of hydrogen.¹⁰ It is noteworthy that the hydrogen bacteria also have the typical normal respiration in addition to oxidation of hydrogen, according to Ruhland.

In the case of the oxidation of methane, a gas with properties much like hydrogen, there is also a large production of energy which is sufficient to bring about chemosynthesis. Then there have also been described bacteria (*B. oligocarbophilus*) which oxidize carbon monoxide to carbon dioxide,¹¹ and also

¹ Molisch, H. Die Eisenbakterien. 1910.

² Lieske, R. Jahrb. wiss. Bot. 49: 91. 1911; 50: 328. 1912; Idem. Centralbl. f. Bakt. (II) 49: 413. 1919. This investigator refutes the results of Molisch who denied the existence of chemosynthesis in the iron bacteria. Cf. also the detailed monograph of Cholodny. Die Eisenbakterien. 1926.

³ Cholodny, N. Ber. bot. Ges. 40: 326. 1922.

⁴ Lieske. Jahrb. wiss. Bot. 50: 328. 1912.

⁵ Beijerinck. Fol. Microbiol. 2: 123. 1913; Söhngen, N. J. Chem. Week. 11: 240. 1914.

⁶ Brussow, A. Centralbl. f. Bakt. (II) 45: 547. 1916; 48: 193. 1918.

⁷ Söhngen. Ibid. 15: 513. 1906; Kaserer. Ibid. 15: 575. 1906; 16: 681 and 769. 1906; Ruhland, W. Jahrb. wiss. Bot. 63: 321. 1924; Grohmann, G. Centralbl. f. Bakt. (II) 61: 265. 1924.

⁸ Söhngen. loc. cit.; Kaserer. loc. cit.

⁹ Ruhland, W. loc. cit. Cf. also: Lebedew, A. v. Biochem. Zeitschr. 7: 1. 1908; Niklewski. Jahrb. wiss. Bot. 48: 113. 1910; Idem. Centralbl. f. Bakt. (II) 20: 469. 1908.

¹⁰ Niklewski. loc. cit.; Ruhland. loc. cit.

¹¹ Kaserer. Centralbl. f. Bakt. 16: 632 and 769. 1906.

use charcoal and peat.¹ Oxidations of other mineral substances may be utilized for the respiration of some bacteria and new discoveries in this field are not precluded. The fact is worth noting that the bacteria with chemosynthesis respire at the expense of mineral substances. The simple oxidations of mineral substances, which are not complicated processes of several stages and which proceed like reactions of ions with an unmeasurably high velocity, appear to be especially well suited to the production of the unusually large quantities of energy which are required for chemosynthesis.

6. THE CYCLE OF NUTRITIVE MATERIALS IN NATURE

All the peculiar chemical transformations just described, which serve as sources of energy for chemosynthesis, also have a general biological significance in that they are responsible for the cycle of the various physiologically important substances in nature. The life of organisms can go on to unlimited numbers of generations only when the feeding of every single organism does not involve the unreplaceable consumption of the food materials in the environment. The following example will illustrate this law. It is known that sulphur is essential for all plants in which it is one unit of which proteins are made. Seed-plants are able to assimilate sulphur only in the form of sulphates. In the plant bodies, however, the sulphur is reduced and fixed in organic compounds. In proteins the sulphur is entirely separated from oxygen, so that the decomposition of the dead plants and animals returns the sulphur to the soil not in the form of oxidized compounds but as hydrogen sulphide. The odor of decaying plant and animal remains depends largely on this hydrogen sulphide. If the transformations of sulphur in nature only took it this far, it would mean that the amount of sulphates on earth would constantly decrease and hydrogen sulphide would accumulate. Since the oxidation of H_2S , other than by biological agencies, takes place too slowly in comparison with the consumption of sulphates by plants, the latter would finally be used up and life on earth would become extinct. Since this is not the case, there must be an equilibrium in the handling of sulphur in nature and all the sulphur reduced in living cells must be changed back to sulphates. The sulphur cycle is completed when the sulphates are reformed so that they can be used again in plant nutrition. The cycle is made possible especially by the sulphur bacteria which make the change from hydrogen sulphide to sulphuric acid.

This discussion of sulphuric acid applies equally well to nitric acid and carbonic acid which are also nutrients for seed-plants. We have already seen that nitrates are reduced in plant cells. Actually there are no organic derivatives of oxidized nitrogen to be found in living cells. When the animals and plants die, the nitrogen is removed chiefly in the form of ammonia. This is also formed by the decomposition of the excrement and urine of animals. The nitrifying bacteria complete the cycle of nitric acid by regenerating it from ammonia and thereby make it available to seed-plants.

¹ Potter, M. C. Proc. Roy. Soc. London (B) 80: 239. 1908.

Carbonic acid must also complete a cycle in which the various kinds of fermentations play important rôles; fermentations usually form simple carbon compounds from the complex ones. These products of fermentation are finally oxidized to carbon dioxide and water which are used again in the nutrition of green plants. Methane represents a product of fermentation and the bacteria which oxidize it to CO_2 and water have a part in preserving the normal equilibrium of carbon in nature. Other bacteria which oxidize simple organic substances play analogous rôles. The cycle of each of the biologically important substances is thus completed through the participation of specific microorganisms. These effect transformations which do not take place in plants with normal nutrition and respiration. There is no other way. To maintain the cycle of any one of these substances it is necessary to have a series of transformations which work in the opposite direction to the physiological changes in the food materials concerned.

It has been mentioned already that chemosynthesis is not the only process which makes possible the cycles of various substances. In different cases, such cycles are completed by organisms which show no signs of chemosynthesis and feed on organic substances. However, such an organism must exhibit a peculiar biochemical specificity that enables it to carry out transformations which are antagonistic to the normal biochemical processes of nutrition. Such biochemical processes in nature complete the cycle of nitrogen, an element that usually occurs in minimum amounts and limits the development of life on earth. We turn now to the consideration of these biochemical processes.

7. FORMATION OF MOLECULAR NITROGEN IN NATURE. DENITRIFICATION

We have already learned (p. 115 ff.) that plants obtain their nitrogen only from the salts of nitric acid and ammonia that are present in the soil and the waters. In plant cells this nitrogen of oxidized nitrogen compounds undergoes reduction. When the plant and animal residues are decomposed, part of the nitrogen enters the bodies of microorganisms living in the soil and the waters, and part becomes ammonia, is changed to nitric acid by means of nitrification and can then be used again to nourish plants. But in this cycle there is a fairly important loss of nitrogen; the nitrogen in the bodies of plants and animals is not all returned directly to the plants because some of the fixed nitrogen is changed to the molecular nitrogen of the atmosphere.

As we know, all compounds of nitrogen dissociate at high temperatures. In the presence of oxygen, ammonia can burn and its hydrogen is oxidized to water while the nitrogen escapes as a gas. In all combustions and slow oxidations most of the nitrogen in the burning organic substance is released in the free form. This is the primary cause of the steady destruction of the small reserve of fixed nitrogen in nature.

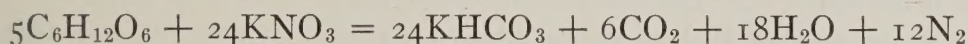
By his own industries, man contributes greatly to this destruction. Not to mention the enormous quantities of combustibles burned in all countries, it is sufficient to point out the destruction of nitrogenous compounds by the use of explosives containing nitrogen. It has been calculated that the amount

of fixed nitrogen returned to the air as molecular nitrogen during the World War 1914-1918, would feed all mankind for many years if it had been used to fertilize the fields.

There are also biological processes which tend to break up nitrogenous compounds to form molecular nitrogen. Among these processes, the so-called denitrification¹ is the most important. The expression "denitrification" is not entirely adequate; it might be thought to include any process antagonistic to nitrification, such as that which changes nitric and nitrous acid to ammonia. Actually it refers to the complete denitrification of nitrates and nitrites, in that the oxygen is used for physiological oxidation and the nitrogen is returned to the atmosphere in the molecular condition.

Denitrification may be either direct or indirect. When it is direct, the process is rapid and free nitrogen is liberated in large quantities. When indirect, the process is slow and involves the formation of nitric oxide and nitrous oxide as well as molecular nitrogen. It is certain that free access to oxygen reduces direct denitrification² because the bacteria respire normally under these conditions. Under anaerobic conditions, however, the oxygen from the nitrites can replace the molecular oxygen for respiration.³ In the case of indirect denitrification, which is brought about by many kinds of bacteria, there is less need for oxygen. Thus the bacteria of denitrification respire like ordinary organisms at the expense of organic substances. Their physiological peculiarity consists in the persistence of their normal oxygen respiration in the absence of molecular oxygen because it continues by using the oxygen of nitrites. There are a great many of these denitrifying bacteria and new species are always being found to have this property.

The fundamental papers of M. Korsakowa⁴ have practically cleared up the chemical side of denitrification. The process of nitrate reduction causes the equalization of the oxidation-reduction potential. Nitrate ions act as hydrogen acceptors; consequently the presence of a hydrogen donator is essential for denitrification. The hydrogen donators are organic substances (sugars, oxyacids) which are thereby oxidized by the nitrate oxygen with the release of energy. Hence there is always a certain quantitative ratio between the amounts of organic substance oxidized and those of the nitrate reduced. If glucose is oxidized, the quantitative relationships are as follows:



It is therefore evident that denitrification, contrary to the current views, can proceed with a low content of organic substances in the soil.⁵

¹ Gayon et Dupetit. *Compt. rend.* 95: 644. 1882.

² Déhérain et Maquenne. *Ann. Agron.* Vol. 5. 1883; Tacke. *Landwirt. Jahrb.* 16: 917. 1888.

³ Weissenberg. *Arch. f. Hyg.* 30: 279. 1897; van Iterson. *Centralbl. f. Bakt. (II)* 12: 107. 1904; Lebedew, A. v. *Schriften der Noworuss. Naturforscherges.* 1911 (Russian).

⁴ Korsakowa, M. *Mitt. d. Akad. Wiss. Leningrad*, p. 1221. 1927; p. 598. 1929; Korsakowa and G. Lopatina. *Ibid.*, p. 507. 1929 (Russian).

⁵ Saltpeter is therefore to be used as a fertilizer with great caution. If it is not absolutely essential for seed-plants, it may result in a considerable infection of the soil with denitrifying bacteria which will reduce its fertility.

The process of denitrification consists of the following steps:

1. $\text{HNO}_3 \rightarrow \text{HNO}_2$
2. $\text{HNO}_2 \rightarrow$ Intermediate product (Nitroxyl HNO or hyponitrous acid $\text{H}_2\text{N}_2\text{O}_2$)
3. Intermediate product $\rightarrow \text{N}_2$

In some cases the nitrate reduction only reaches the stage of nitrous acid or oxide of nitrogen. The formation of ammonia which takes place in other cases branches off from step 2.

Some organisms of fermentation are also able to reduce nitrates but the end-product is only HNO_2 in most cases. Facultative anaerobes are able to activate to donators the substances contained in the solution including the products of fermentation. In this connection formic acid, for example, is more easily activated than acetic acid.

Some organisms obtain their working energy not only from nitrites but also from chloric acid, arsenious acid and potassium ferrocyanide.¹

8. ASSIMILATION OF MOLECULAR NITROGEN BY MICROÖRGANISMS WHICH LIVE IN THE ORGANS OF SEED-PLANTS

There must be an unlimited number of natural phenomena which counteract the release of molecular nitrogen yet the formation of molecular nitrogen probably exceeds the decomposition of nitrogenous compounds. It must be borne in mind that during the earlier period of our earth, when it had just cooled, no nitrogenous compounds could exist on the earth's surface because they dissociate at high temperatures. Very little nitrogen is oxidized in the air by electrical discharges so we know that most of the fixed nitrogen now on the earth's surface is the result of biological processes. We actually know of different microbes which assimilate the molecular nitrogen of the atmosphere and new organisms with this capacity are always being described.

The first of these cases to be discovered was the assimilation of nitrogen by the roots of the *Leguminosae*—a process which is important in agricultural practices. It was known even to the ancients that the alternation of grains and legumes was beneficial to the crops of these plants. Pliny discussed the subject. After experiment stations and test plots were established, it was learned definitely that the growth of *Leguminosae* increased the supply of nitrogen in the soil.² At the start of his scientific career, Boussingault³ supposed that the *Leguminosae*, unlike other plants, are able to assimilate atmospheric nitrogen but when he undertook to prove this by experiments in which the soil was replaced by sand sterilized by heat and watered with definite solutions, this famous agricultural chemist obtained the opposite, clean-cut result that the nitrogenous nutrition of *Leguminosae* is no different from that of other crop plants. The objections⁴ to this conception soon proved to be invalid.

¹ Ampola, G. e Ulpiani. Gazz. Chim. Ital. 28: 410. 1898; Idem. Chem. Centralbl. (II) for 1898, p. 896.

² Lawes and Gilbert. Jour. Chem. Soc. (London) 47: 380. 1886; and others.

³ Boussingault. Compt. rend. 6: 102. 1837; 7: 889. 1838; Idem. Ann. chim. et physique (2) 67: 1. 1837; 69: 353. 1838.

⁴ Ville. Compt. rend. 36: 464 and 650. 1852; 38: 705 and 723. 1854.

In this way there arose a complicated problem: on the one hand experiments in the open showed clearly that the *Leguminosae* grow very well on soils poor in nitrogen but, on the contrary, it was impossible by exact laboratory experiments to confirm the observations of the experiment stations.¹

This problem was solved by the renowned investigations of Hellriegel and his co-workers.² His first results seemed to contradict the classical experiments of Boussingault (p. 115): *Leguminosae* develop normally in the absence of soil nitrogen while the *Gramineae* gave the opposite results (Figs. 24 and 25). Further investigations explained the cause of this apparent contradiction. There is a noticeable difference between *Gramineae* and *Leguminosae* only in unsterilized soils. If the natural soil is replaced by sterilized sand, as Boussingault performed the experiment, there is no difference in the behavior of the grasses and the legumes; the latter fail to grow well in the absence of fixed nitrogen.

Hellriegel failed to observe that the roots of the *Leguminosae* are usually provided with characteristic little tubercles, the shape of which differs for the various species. It has been found that these root tubercles occur only under non-sterile conditions. No tubercles form in sterilized sand and the plants are unable to grow well without fixed nitrogen. It is sufficient to water the sterilized soil with an extract of natural soil in which the legume to be studied has been grown, in order to produce a good growth of tubercles on the roots of the experimental plants, and to give the plants the capacity to fix nitrogen. The following table of Hellriegel and his co-workers illustrates these results. In the absence of compounds of nitrogen, the various plants showed the following dry weights and increases in nitrogen at the end of the experiment (3 months from seed); cf. also Figs. 24 and 25.

Name of plant	Grown in sterile sand		Grown in sterile sand plus soil extract	
	Dry wt. of aerial parts in grams	Increase in N in mg.	Dry wt. of aerial parts in grams	Increase in nitrogen in mg.
Oats.....	0.671	±0	0.659	-1
Barley.....	0.586	-1		
Buckwheat.....	0.060	±0	0.040	±0
Seradella.....	0.062	±0	18.190	+395
Lupine.....	0.921	-8	42.451	+1286
Peas.....	0.928	-2	20.426	+473

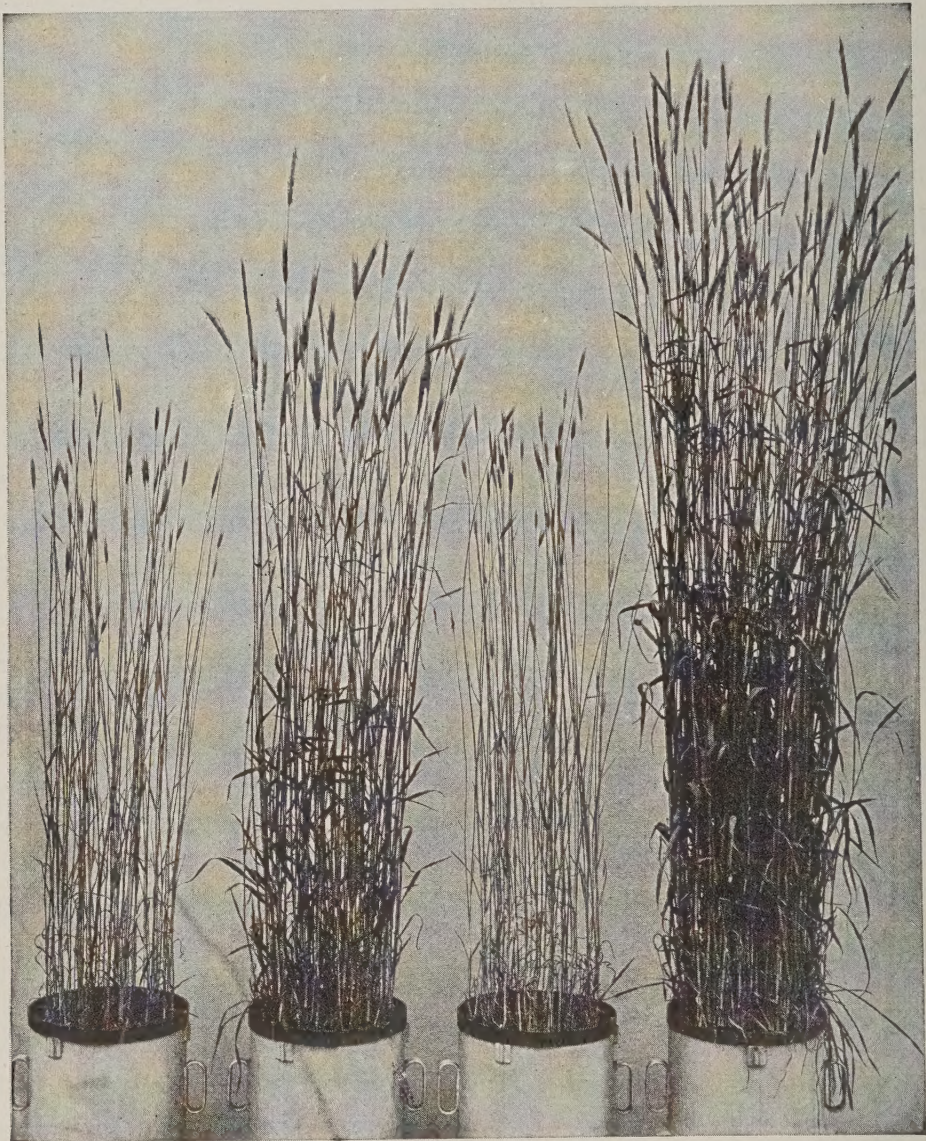
The *Leguminosae* therefore gain in nitrogen on soils lacking nitrogen if the characteristic tubercles have developed on their roots. The gain in nitrogen is obviously possible at the expense of molecular nitrogen of the air. The fore-

¹ A favorite saying of unscientific men explains it thus: "Theoretically it is true but it doesn't work out in practice." However, the whole history of science shows that theory and practice disagree only in fields which are insufficiently investigated. Such a discrepancy is a direct hint of the possibility of new discoveries.

² Hellriegel, H. und H. Wilfarth. Untersuchungen über die Stickstoffnahrung der Gramineen und Leguminosen. 1888.

sighted experiments of Schloesing and Laurent,¹ which constitute a fitting supplement to Hellriegel's experiments, showed from gas analyses that the gain in nitrogen by *Leguminosae* in the course of 3 months corresponds exactly to the nitrogen deficit of the atmosphere:

	N in air of container at start of exp.	N in air of container at close of exp.	N lost from air	N gained by plants
Peas No. 1	2681.2 cc.	2651.1 cc.	29.1 cc. = 36.5 mg.	40.6 mg.
Peas No. 2	2483.3 cc.	2457.4 cc.	25.9 cc. = 32.5 mg.	34.1 mg.



1. 2. 3. 4.
FIG. 24.—Influence of mineral fertilizers on *Gramineae*. From left to right: 1. Without fertilizer; 2. NaNO_3 ; 3. K and P; 4. K, P and NaNO_3 . A normal crop is obtained only by using saltpeter. (After Wagner.)

Cultures of *Leguminosae* free from tubercles, like cultures of other plants, showed neither loss of nitrogen from the air nor gain in nitrogen by the plants. This demonstrated an assimilation of atmospheric nitrogen by direct analyses.

¹ Schloesing et Laurent. Compt. rend. 111: 750. 1890; 113: 776. 1891; 115: 881 and 1017. 1892; Idem. Ann. Inst. Pasteur. 6: 65 and 824. 1892.

The exact work of Hellriegel and his colleagues, at once confirmed by Lawes and Gilbert,¹ solved the question of the nitrogenous nutrition of the legumes in principle. It only remained to explain the rôle of the root tubercles, the presence of which makes it possible to assimilate molecular nitrogen. By sectioning a tubercle (Fig. 26, *b* and *c*) it can be seen that the inner portion consists of cells filled with protoplasm. In these there are remarkable, peculiarly branched structures (Fig. 26, *e* and *f*) which have the appearance of strange



FIG. 25.—Influence of mineral fertilizers on *Leguminosae*. From left to right: 1. Without fertilizer; 2. NaNO_3 ; 3. K and P; 4. NaNO_3 , P and K. The addition of saltpeter has no effect. (After Wagner.)

inclusions. Earlier they were thought by some investigators to be normal constituents of the protoplasm,² by others to be parasitic fungi³ but long ago they were correctly declared by Woronin⁴ to be bacteria. The form of the branched structures, called bacteroids, is very different from the forms of typical bacteria but they resemble the so-called involution forms, *i.e.*, malformations, which are characteristic of bacteria under unfavorable conditions. To be sure, some workers⁵ deny that bacteroids are simple involution forms.

¹ Lawes and Gilbert. Proc. Roy. Soc. London. 43: 108. 1887; 47: 85. 1890; Idem. Phil. Trans. Roy. Soc. London (B) 180: 1. 1889.

² Brunchorst. Ber. bot. Ges. 3: 241. 1885; Tschirch. Ibid. 5: 58. 1887; Frank. Ibid. 7: 332. 1889.

³ Ward, M. Phil. Trans. Roy. Soc. London. 178: 173. 1886.

⁴ Woronin, M. Mitt. d. St. Petersburg. Akad. d. Wiss (7) Vol. 10, No. 6. 1866 (Russian).

⁵ Hiltner. Centralbl. f. Bakt. (II) 6: 273. 1900; Neuman. Landwirt. Versuchs-Stat. 56: 189. 1901; and others. Stefan (Centralbl. f. Bakt. (II) 16: 143. 1906, and others) considers bacteroids to be involution forms.

Beijerinck¹ finally obtained pure cultures of bacteria from the tubercles of the legumes. When grown on artificial media they change from the structures isolated from the tubercles into the normal rod-like bacteria (Fig. 26, *d*). The species of bacteria which Beijerinck called tubercle bacteria (*Bacillus radicola*) stands in the classification near the group *Bact. pneumoniae*—the bacteria which produce inflammation of the lungs. The members of this group are apparently engaged in the assimilation of molecular nitrogen.² For a long time

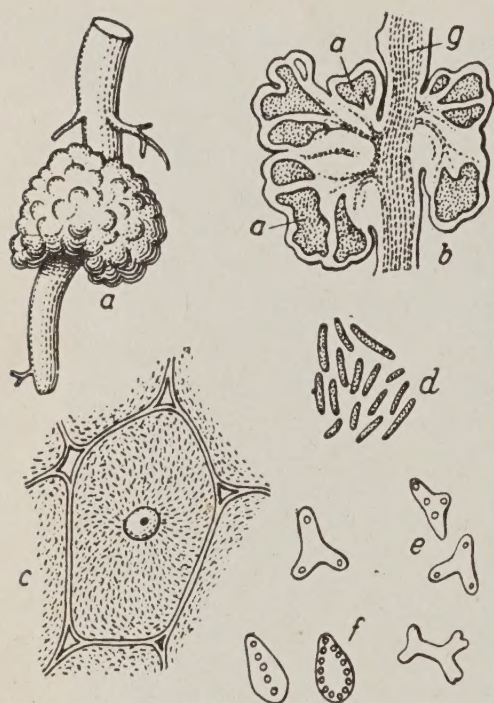


FIG. 26.—The root tubercles of *Leguminosae*: *a*. Tubercle of lupine (natural size); *b*. Longitudinal section of this tubercle (somewhat enlarged), *g* = vascular bundle, *a* = tissue containing bacteroids; *c*. A cell of the bacteroid tissue, filled with bacteria (magnified 600 $\times$); *d*. Typical form of tubercle bacteria; *e*. Bacteroids of vetch; *f*. Bacteroids of lupine. (*d*, *e* and *f* are enlarged 500 $\times$.) (After A. Fischer.)

the origin of the tubercle was a riddle which was finally solved by the excellent work of Prazmowski.³ He started cultures of *Leguminosae* in sterilized sand which lacked the microorganisms of the soil, and then inoculated the sand with a pure culture of tubercle bacteria. The bacteria enter the plants through the root hairs.^m By secreting substances which dissolve the cell wall of the root hair,⁴ the bacteria enter the protoplasm and the tip of the root hair curls. At this time the bacteria secrete a sheath about themselves (the ordinary protective device of parasitic microorganisms) and in this condition reach the parenchyma of the cortex. Here the bacteria shed their coverings and come into direct contact with the protoplasm of the cell and in a short time turn into bacteroids. This change in form can be obtained under artificial conditions by providing the bacteria with an excess of carbohydrates or organic acids.⁵

The cells of the cortex which are filled with bacteroids begin to divide rapidly. Finally there is formed a tubercle provided with special conductive tissues which provide for

an interchange of materials between the tubercle and the other parts of the plant.

At first the working out of the problem of the nitrogenous nutrition of the *Leguminosae* was very successful and a series of excellent experimental investigations were described in print; later real obstacles were encountered. Many

¹ Beijerinck. *Böt. Zeit.* 46: 725. 1888.

² Löhnis. *Centralbl. f. Bakt. (II)* 14: 596. 1905.

³ Prazmowski. *Landwirt. Versuchs-Stat.* 37: 161. 1890; 38: 5. 1891.

^m It has been shown by Thornton (*Proc. Roy. Soc. London (B)* 104: 481-492. 1929) that the bacteria do not enter the root hairs of *Medicago sativa* until the first leaves of the seedling open. The delay is caused by the plants and the presence of older plants starts the infection sooner. The action seems to be that of a root secretion which hastens the growth of the bacteria.—*Ed.*

⁴ Hiltner. *Arb. d. biol. Abt. d. kaiser. Gesundheitsamtes.* 1: 180. 1900.

⁵ Hiltner. *Lafars Mykol.* 3: 51.

workers were unable to find a fixation of molecular nitrogen by means of pure cultures of the tubercle bacteria or by tubercles removed from the plants, so the question arose whether, under the influence of the bacteria, the legumes themselves did not have the power to assimilate atmospheric nitrogen through their leaves. However, direct experiments show that the nitrogen is fixed by the roots and not by the aerial portions of the plants.¹ Some investigators think that the tubercle bacteria do not assimilate the molecular nitrogen but only have the capacity to remove the least traces of fixed nitrogen compounds from the soil. Others have ascribed the gain in nitrogen by legumes to the fact that they utilize the nitrogen of the bacteroids and thus parasitize them, so to speak. This hypothesis is hardly probable because the amount of nitrogen within the bacteroids is really insufficient. Later some workers have observed the assimilation of molecular nitrogen by alleged pure cultures of tubercle bacteria² but their results are not valid for various reasons. Beijerinck himself, who described an assimilation of nitrogen by tubercle bacteria to the extent of 1-1.8 mg. from 100 cc. of a 1.5-2% sugar solution in an extract of legume seedlings, later denied the capacity of tubercle bacteria to assimilate molecular nitrogen.³ The later investigations of Barthel, which were carried out with faultlessly pure cultures, led to the conclusion that tubercle bacteria are incapable of fixing nitrogen either in the normal form or in the form of bacteroids outside of seed-plants.⁴

On the other hand, after many unsuccessful experiments, the fixation of nitrogen by whole tubercles was finally established experimentally.⁵ It can be said then that even though only negative results have been obtained for the problem of assimilation of molecular nitrogen by means of isolated tubercle bacteria, we must believe that the *Leguminosae* are able to use atmospheric nitrogen for their nourishment because of the tubercle bacteria which live in their roots. A valid solution of the problem still remains to be given.

Experiments in inoculating plants of other families with the tubercle bacteria have not given favorable results.⁶ This is quite conceivable because there is a specific adaptation for certain forms of tubercle bacteria to be found even within the legume family. Thus the following results are obtained by inoculations with bacteria from other species:⁷

With bacteria from	<i>Robinia</i>	<i>Vicia</i>	<i>Pisum</i>
<i>Robinia</i>	232.1	13.5	21.2
<i>Vicia</i>	12.9	264.0	22.6

Thus each species of plants yields an abundance of fixed nitrogen only with its own bacteria. Inoculations with alien bacteria often produce no tubercles but some substitutions will work. Thus peas, beans, clover and lucerne assimi-

¹ Kossowitsch, P. The assimilation of free nitrogen by plants. 1895 (Russian); Nobbe und Hiltner. Landwirt. Versuchs-Stat. 52: 455. 1899.
² Beijerinck. Verslagen Med. d. Akad. v. Wetensch. Amsterdam (3) 8: 469. 1891; Mazé. Ann. Inst Pasteur. 11: 44. 1897; 12: 1. 1898; Golding. Jour. Agr. Sci. 1: 59. 1905; and others.
³ Beijerinck. Med. kon. Akad. d. Wet. Amsterdam. 26: 1456. 1918.
⁴ Barthel, Chr. Med. 308 från Centralanstalten får försöksväsendet på jordbruksområdet. 1926. See also Löhnis, M. P. Soil Sci. 29: 37-57. 1930.]
⁵ Krascheninnikoff. Anniv. vol. for K. A. Timiriazeff, p. 307. 1916.
⁶ Stutzer, Burri und Maul. Centralbl. f. Bakt. (II) 2: 665. 1896; Grossbüsch. Diss. Bonn. 1907.
⁷ Nobbe, Hiltner und Schmid. Landwirt. Versuchs-Stat. 45: 1. 1894.

late nitrogen very well by the use of the same [strain of] bacteria.¹ The opposite results are found for other plants and the conditions are suggestive in connection with the "equilibrium" between the legumes and their tenants. When roots are inoculated in the middle of the summer, no bacteroids are formed; the tubercle bacteria then behave like typical parasites and the legumes are only injured by their entrance.² In this case the balance of power is too much on the side of the microörganism. On the contrary, vigorous specimens of *Leguminosae* often digest the bacteria which penetrate their tissues³ and no tubercles are formed even though it is to the disadvantage of the plant. Here the balance is on the side of the legumes.

A series of observations thus show that there is a constant strife between the *Leguminosae* and the tubercle bacteria and that only equal strength of the two organisms gives an equilibrium by which both profit. When growth stops in the autumn the tubercles are broken up and the bacteria enter the soil *in much greater numbers* as compared with those which entered the plants. This provides for a continued increase in numbers of the bacteria through an unlimited series of generations. On the other hand, the *Leguminosae* require no nitrogen from the soil. Direct experiments show that even with an abundance of nitrogenous fertilizers most of the nitrogen assimilated by the *Leguminosae* is taken from the atmosphere. For example, when *Vicia villosa* is not given nitrogen fertilizer it takes 93.8% of its nitrogen from the atmosphere and when it is well fertilized with saltpeter, 73% of all the nitrogen found in the plant enters it from the air.⁴ We shall return later to the relations between the *Leguminosae* and their bacteria.

Tubercles containing bacteria are also found in the roots of some plants in other families, such as *Eleagnus*, *Rheum*, *Alnus*⁵ and *Casuarina*.⁶ With regard to the alder, the literature contains statements to the effect that it develops at the expense of atmospheric nitrogen when it has tubercles.⁷ Analogous facts have been published with respect to *Podocarpus* which also has root tubercles.⁸ It is interesting that these tubercles are inhabited by a fungus in place of bacteria. Some of the tropical *Rubiaceae* and *Myrsinaceae* [such as *Ardisia crispa* and species of *Pavetta*] have bacterial tubercles on the leaves⁹

¹ There is an abundance of literature on this point. Cf. e.g., Laurent. Ann. Inst. Pasteur. 5: 119. 1891; Nobbe, Schmid, Hiltner und Hotter. Landwirt. Versuchs-Stat. 39: 345. 1891; Déhérain et Demoussy. Compt. rend. 130: 20. 1900; Nobbe und Hiltner. Landwirt. Versuchs-Stat. 42: 468. 1893; and many others [such as the paper by Löhnis and Leonard. U. S. Dept. of Agr. Farmers' Bull. No. 1496. 1926 which also gives practical directions for the use of these bacteria].

² Beijerinck. Bot. Zeit. 46: 726 and 790. 1888; van der Wolk. Cultura, vol. 28, No. 336. 1916; and many others.

³ Hiltner. Centralbl. f. Bakt. (II) 10: 479. 1903.

⁴ Nobbe und Richter. Landwirt. Versuchs-Stat. 59: 167. 1904.

⁵ Woronin, M. Mitt. d. russ. Akad. d. Wiss. Vol. 10, No. 6. 1886; Möller. Ber. bot. Ges. 3: 102. 1885; Bottomley. Ann. Bot. 26: 111. 1912; Spratt. Ibid. 26: 119. 1912.

⁶ Miehe, H. Flora. 111/112: 431. 1918.

⁷ Nobbe, F. und L. Hiltner. Naturwiss. Zeitschr. f. Landwirt. und Forstwesen. 2: 366. 1904; Hiltner. Landwirt. Versuchs-Stat. 46: 153. 1895; Möller. Zeitschr. f. Forst- und Jagdwesen 44: 527. 1912.

⁸ Nobbe, F. und L. Hiltner. Landwirt. Versuchs-Stat. 51: 241. 1899.

⁹ Zimmermann. Jahrb. wiss. Bot. 37: 1. 1902; Faber. Ibid. 51: 285. 1912; 54: 243. 1914; Miehe. Ber. bot. Ges. 29: 156. 1911; Idem. Chem. Zeit. 36: 1110. 1912; Idem. Jahrb. wiss. Bot. 53: 1. 1913; 58: 29. 1917; Idem. Ber. bot. Ges. 34: 576. 1916; also Georgewitsch, P. Bull. Roy. Bot. Gar. Kew for the year 1916, p. 105 (Kraussia).

and the bacteria which form the tubercles can be found in the seeds. Not only do the bacteria feed their host plants with atmospheric nitrogen but they also play a very important rôle in the development of the host from its seed. In the absence of bacteria the latter develops abnormally and pathologically.

From the foregoing statements it is evident that the symbiosis of seed-plants and organisms which fix nitrogen is a rather common phenomenon and more analogous facts will possibly be discovered in the future.

9. ASSIMILATION OF MOLECULAR NITROGEN BY FREE-LIVING BACTERIA

It has long been known to agricultural practice that the crop is larger on a soil containing an abundance of microorganisms. Direct statements of the increase in nitrogen in these soils have also been made. This led to the hypothesis that some of these organisms in the soil could fix molecular nitrogen.

The first of these organisms was isolated by Winogradsky by the use of his method of elective cultures.¹ A nutrient solution containing no nitrogen was inoculated with a small amount of field soil. Most of the microorganisms did not even grow in it and a lively butyric acid fermentation took place in the liquid (Chap. VIII).

Three species of bacteria were isolated from elective cultures, of which two proved to be ordinary saprophytes. The third was a form producing butyric acid fermentation but it also had the capacity to fix molecular nitrogen. This species, which belongs to the spore-forming genus *Clostridium* and was given the name *Clostridium Pasteurianum* (Fig. 27), is an obli-

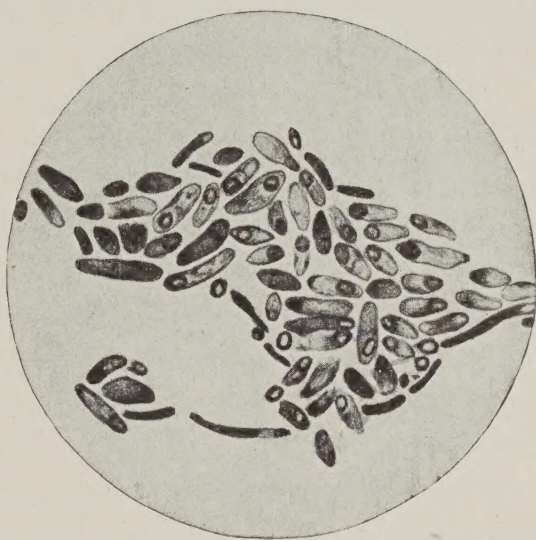


FIG. 27.—*Clostridium Pasteurianum*. Cells with spores. Enlarged 1000 X. (After Omeliansky.)

gate anaerobe, *i.e.*, it can grow only in the absence of oxygen. On the one hand, it needs air because it feeds on atmospheric nitrogen but on the other hand it would suffer from the toxic effect of oxygen. Under natural conditions this difficulty is overcome by a symbiosis with other bacteria. *Clostridium Pasteurianum* is always accompanied by some saprophytic bacteria which can grow at the expense of minimum quantities of fixed nitrogen. They absorb all the oxygen in the vicinity of *Clostridium Pasteurianum* and make it possible for the spores of this organism to germinate. It is evident that such a bacterial partnership can exist in almost complete absence of nitrogenous compounds. The more serious risk for most plants—nitrogen hunger—does not exist for this partnership. It is very interesting and instructive to observe their development in an open vessel which contains no nitrogenous substances and which is inoculated with a mixture of *Clostridium Pasteurianum* and the forms which

¹ Winogradsky, S. Compt. rend. 116: 1385. 1893; 118: 353. 1894; Idem. Arch. f. biol. Wiss. 3: 297. 1895; Idem. Centralbl. f. Bakt. (II) 9: 43. 1902.

accompany it. At first the bacterial growth is slow. It all depends on that of the saprophytic form which obtains enough nitrogen in the form of impurities to grow fast enough to remove the oxygen about the *Clostridium*. This first period is the weakest in the life of our partnership. As soon as the *Clostridium* has increased somewhat, conditions change at once. The development of all the members of the partnership is rapid because the form which accompanies the *Clostridium* obtains enough nitrogenous foods to multiply rapidly and make it possible for the *Clostridium* to develop freely. If enough nitrogenous foods have accumulated in the liquid, various other organisms appear in it because they enter from the air and find there a favorable medium on which to grow. If the liquid is placed in the light, various algae will appear in it and the occupants of the dish will then be continually supplied with carbon as well as nitro-

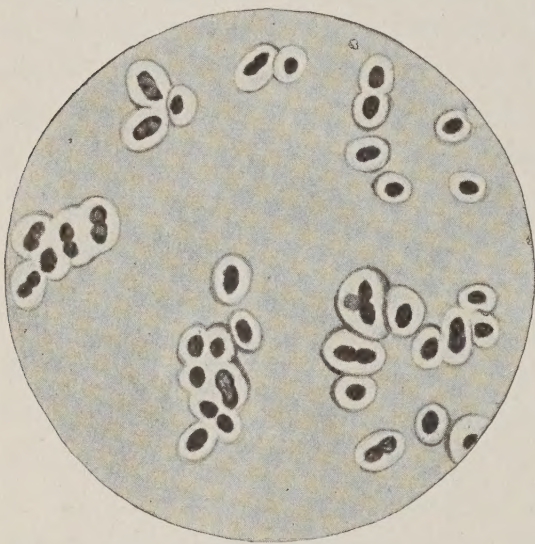


FIG. 28.—*Azotobacter chroococcum*. Enlarged 1000 $\times$. (After Omeliansky.)

gen. The growth will then be limited only by lack of space. This little experiment shows the way life develops in desert regions where the necessary nitrogenous substances for plant nutrition are lacking.

The source of energy for *Clostridium Pasteurianum* is not oxidation but butyric acid fermentation (Chap. VIII). In the pure cultures of the earlier workers, *Clostridium Pasteurianum* assimilated 3–8 mg. of nitrogen for each gram of sugar fermented. Such an output is possible only when the substrate contains very little nitrogen. When fed freely with ammonium salts, the organism does not assimilate molecular nitrogen.

Another common form which is able to utilize molecular nitrogen, was discovered by Beijerinck¹ and named *Azotobacter*. Two species are usually recognized: *Azotobacter chroococcum*, non-motile form, and *A. agile* which is motile and fluorescent.² They are interesting in many respects; they are quite large for bacteria (Fig. 28), are colored and exhibit an extremely high rate of respiration. The material for this respiration is probably various sugars in the soil, where they arise as cleavage products of plant residues, especially cellulose and pectins. *Azotobacter* is often found in symbiosis with algae, particularly with the *Cyanophyceae*.³ The algae provide the bacteria with sugar and use the nitrogen compounds prepared by the bacteria. Since organic substances both with and without nitrogen are thus obtained from the atmosphere, the symbiosis of *Cyanophyceae* with *Azotobacter* guarantees the welfare of both organisms. It is interesting that light is important for the fixation of nitrogen by *Azotobacter agile* and the less refrangible rays are said to have more

¹ Beijerinck. Centralbl. f. Bakt. (II) 7: 567. 1901.

² Other authors recognize about 20 species of *Azotobacter*. Cf. Löhnis, F. und T. Westermann. Centralbl. f. Bakt. 22: 234. 1908. *Azotobacter* varies greatly in color.

³ Fischer, H. Ibid. 12: 267. 1904; Reinke, J. Ber. bot. Ges. 21: 378 and 481. 1903.

effect than the more refrangible ones.¹ The bacterial fluorescence is green. The species of *Azotobacter* are found in various soils and in both fresh and salt water.²

In addition to the two most important and typical forms, *Clostridium Pasteurianum* and *Azotobacter*, other bacteria have been found which are said to be able to assimilate molecular nitrogen.³ They belong in part to the *Clostridium* type and in part to the *Azotobacter* group. Most of the organisms of the *Clostridium* type have been found to be varieties of the single species, *Bacillus amylobacter*.⁴ Other N-fixing bacteria are strains of *Bacillus astero-sporus*⁵ which grow in the presence of air. All these organisms are extremely common in nature.⁶ The quantities of nitrogen assimilated by them fluctuate over a very wide range according to the prevailing conditions.

The free-living soil bacteria which accompany *Azotobacter*, such as *Bact. aerogenes*, *Bact. radiobacter*ⁿ and forms near these, are also alleged nitrogen fixers.⁷ Apparently this capacity is peculiar to all members of the group *Bact. pneumoniae* (see above).

10. ASSIMILATION OF MOLECULAR NITROGEN BY FUNGI AND ALGAE

Some investigators have observed a slight assimilation of molecular nitrogen by molds but these results have not been confirmed for the most part. Positive results have been obtained with *Aspergillus niger* and *Penicillium glaucum*,⁸ and with some fungi that have been isolated from dead leaves⁹ and from the root of some *Ericaceae* and *Vacciniaceae*.¹⁰ Positive results were first obtained not long ago with the fungus *Phoma Betae*.¹¹ For other positive results just referred to, the amounts of assimilated nitrogen observed lie within the unavoidable experimental errors; on the other hand, the negative results in this field must be accepted with some reserve because they have been obtained in laboratory experiments with organisms which certainly might fix nitrogen. In every case only fungi freshly isolated from the soil should be used for experiments or the fungus culture should first be placed underground. This precaution was

¹ Kayser, E. Compt. rend. 171: 969. 1920; 172: 183, 491 and 939. 1921.

² Gerlach und Vogel. Centralbl. f. Bakt. (II) 8: 673. 1902; Benecke und Keutner. Ber. bot. Ges. 21: 338. 1903; and others.

³ Perotti. Ann. di. botanica. 4: 213. 1906; Volpino. Centralbl. f. Bakt. (II) 15: 70. 1906; Pringsheim. Ibid. 31: 23. 1911; Truffaut, G. et N. Bezssonoff. Compt. rend. 175: 544. 1922.

⁴ Bredemann. Ber. bot. Ges. 26a: 362. 1908; Idem. Centralbl. f. Bakt. (II) 23: 385. 1909.

⁵ Bredemann. Ibid. 22: 44. 1908.

⁶ *Clostridium Pasteurianum* has been found in almost all the soils of Russia. Species of *Azotobacter* are common in southern loams and in humus carbonate soils. Cf. Arbeiten d. Bureaus f. landw. Mikrobiologie. Vols. 1-3. 1926-1928. Mechanical disturbance of the soil checks the work of *Clostridium Pasteurianum* but facilitates that of *Azotobacter*.

ⁿ According to Löhnis and Hansen (Jour. Agr. Research. 20: 543-557. 1921) *Bacillus* (= *Bacterium*) *radiobacter* is also "regularly present in the root nodules of leguminous plants, stimulating development and activity of the nodule bacteria" which it sometimes outnumbered.—Ed.

⁷ Löhnis. Centralbl. f. Bakt. (II) 14: 593. 1905.

⁸ Berthelot. Compt. rend. 116: 842. 1893; Puriewitsch. Ber. bot. Ges. 13: 342. 1895; Saida. Ibid. 19: 107. 1901.

⁹ Froelich. Jahrb. wiss. Bot. 45: 256. 1908; Stahel, G. Ibid. 49: 579. 1911.

¹⁰ Ternetz, Charlotte. Ibid. 44: 353. 1907; Rayner, M. C. Ann. Bot. 29: 97. 1915; Idem. Bot. Gaz. 73: 226. 1922.

¹¹ Duggar, B. M. and A. R. Davis. Ann. Missouri Bot. Gard. 3: 413. 1916.

neglected by all investigators who report negative results.¹ The one piece of work in which the assimilation of immense quantities of molecular nitrogen by the mold *Aspergillus niger* is described² is undoubtedly based on erroneous data.³ All writers point out that molds assimilate atmospheric nitrogen well only when they are fed with some fixed nitrogen at the same time.

It is interesting that the representatives of the *Actinomycetes* or ray fungi, which grow in the soil and give off the characteristic odor of fertile earth, are unable to assimilate atmospheric nitrogen.⁴ Negative results are obtained even after underground storage and by growing them on soil extracts and other media especially favorable to the fixation of nitrogen.⁵ To summarize all the statements in the literature concerning the fixation of nitrogen by fungi (which have not been thoroughly covered here), it appears that some representatives of this group are able to fix small amounts of nitrogen.⁶ New, thorough investigations are needed for the others. It would be very interesting to experiment with various mycorrhiza and especially with Basidiomycetes. Perhaps new and unexpected results can be had in this field by working with fungi freshly isolated from soil and by mixing them with humus or humus extracts (Chap. VII).

It is still more difficult to obtain reliable results with algae on account of the inadequate methods of growing them in pure cultures. Undoubtedly the *Cyanophyceae* and the green algae assimilate the atmospheric nitrogen in the absence of nitrogen compounds.⁷ However, this behavior has been established with impure cultures; now we know that algae are often found in symbiosis with bacteria which fix nitrogen. Different workers have obtained negative results with pure cultures of green algae.⁸ The later, detailed investigation by Wann,⁹ who reported positive results with single-celled algae, was refuted by the subsequent experiments of Bristol and Page.¹⁰ For practical purposes, a symbiosis between green algae and nitrogen-fixing bacteria is of great advan-

¹ Czapek, F. Hofmeisters Beitr. z. chem. Physiol. und Path. 2: 557. 1902; Gerlach und Vogel. Centralbl. f. Bakt. (II) 10: 641. 1903 (these investigators obtained a slight fixation of nitrogen); Kossowicz, A. Biochem. Zeitschr. 64: 82. 1914.

² Latham, M. E. Bull. Torrey Bot. Club. 36: 235. 1909.

³ Kostytschew, S. and E. Tswetkova. Jour. russ. bot. Ges. 6: 19. 1921 (Russian). Cf. also Pennington, L. H. Bull. Torrey Bot. Club. 38: 135. 1911.

⁴ Beijerinck. Centralbl. f. Bakt. (II) 6: 7. 1900; Gerlach und Vogel. Ibid. 10: 641. 1903.

⁵ Korsakowa, M. and S. Kostytschew. An unpublished paper.

⁶ Duggar, B. M. and A. R. Davis. Ann. Missouri Bot. Gard. 3: 413. 1916 obtained for *Phoma Betae* a nitrogen fixation of 1.8–7.6 mg. and for *Azotobacter chroococcum* of 16.9–17.6 mg. on 100 cc. of nutrient solution. [See also the summary by Senn, G. Biol. Rev. and Biol. Proc. Cambridge Phil. Soc. 3: 77–91. 1928.]

⁷ Schloesing et Laurent. Ann. Inst. Pasteur. 6: 65 and 824. 1895; Idem. Compt. rend. 115: 733. 1892; Kossowitsch, P. Bot. Zeit. 52: 112. 1894; Idem. The assimilation of free nitrogen by plants. 1895 (Russian); Bouilhac. Compt. rend. 123: 828. 1896; Bouilhac et Giustiniani. Ibid. 137: 1274. 1903; Stoklasa. Centralbl. f. Bakt. (II) 6: 22. 1900; Heinze. Ibid. 16: 705. 1906; Idem. Landwirt. Jahrb. 35: 895. 1906; Moore, B. and A. Webster. Proc. Roy. Soc. London (B) 91: 201. 1920; Moore, B. and E. Whitley. Ibid. 92: 51. 1921. [Drewes has reported recently (Centralbl. f. Bakt. (II) 76: 88–101. 1928) that his pure cultures of species of *Anabaena* and *Nostoc* fix nitrogen at the rate of 2 or 3 mg. of N per 250 cc. of nutrient solution in 2 months.]

⁸ Kossowitsch, P. loc. cit.; Schloesing and Laurent. loc. cit.; Krüger, W. und W. Schneidewind. Landwirt. Jahrb. 29: 771. 1900; Charpentier, P. G. Ann. Inst. Pasteur. 17: 321. 1903; Schramm, J. Ann. Missouri Bot. Gard. 1: 157. 1914.

⁹ Wann, F. B. Amer. Jour. Bot. 8: 12. 1921.

¹⁰ Bristol, B. M. and H. J. Page. Ann. Appl. Biol. 10: 378. 1923.

tage; such a partnership feeds entirely from the air because the necessary mineral substances are obtained in sufficient amounts from the dust of the air while nitrogen and carbon are assimilated from the gaseous molecular nitrogen and carbon dioxide respectively.

When Treub¹ visited the isle of Krakatau after the famous volcanic eruption, he found it was first occupied by the *Cyanophyceae*. As the result of their power, either alone or in symbiosis with N-fixing bacteria, “to feed from the air,” the *Cyanophyceae* are pioneers of the plant world on virgin, still unfertile soils.

II. THE CONDITIONS AND THE QUANTITATIVE SIDE OF NITROGEN FIXATION

All investigators who have dealt with the question of nitrogen fixation refer to the great variations in the rate at which the N-fixing microbes work. In the experiments of the earlier workers, *Clostridium Pasteurianum* fixed 2–5 mg. of nitrogen per gram of sugar fermented. With *Azotobacter*, Krzemieniewsky² obtained a gain in nitrogen of 2.4–17 mg. for each gram of sugar used in respiration. This investigator proved by exhaustive experiments that the nitrogen fixation by *Azotobacter* is commonly stimulated by the presence of sterilized natural humus or even garden soil. Soil extracts are considerably less effective than fertile soil. The nature of the stimulating effect of soil or soil extracts has not yet been explained. For *Azotobacter*, good aeration is very important.³

After being grown for some time on artificial nutrients, *Clostridium Pasteurianum* loses the capacity to fix nitrogen but it is regenerated by underground storage.⁴ On the contrary, *Azotobacter* does not lose the capacity to fix nitrogen on artificial media.⁵ A considerable increase in N-fixation is also obtained in some cases by growing two or more species in the same culture. The importance of the symbiosis of N-fixing bacteria and algae was considered above. In other cases the reciprocal use of by-products may exert a favorable influence on the fixation of nitrogen. However, it is to be noted expressly that this influence of joint culture does not always appear.⁶ A generous feeding of nitrogen in the form of inorganic salts has a depressing effect on the fixation of nitrogen but organic nitrogenous compounds have no effect.⁷ Winogradsky⁸ found that *Clostridium Pasteurianum* assimilates free nitrogen less vigorously in proportion to the fixed nitrogen contained in the substrate in the form of NH₃. For example, *Clostridium* fixes the following quantities of nitrogen in the presence of different amounts of NH₃:

N in mg. provided as (NH ₄) ₂ SO ₄	2.1	4.2	6.4	8.5	17.0	21.0
N in mg. assimilated from N ₂	7.0	5.0	5.5	3.6	trace	0

¹ Treub, M. Ann. jard. bot. Buitenzorg, Vol. 7. 1888.
² Krzemieniewsky, S. Bull. acad. sci. Cracovie for 1908. P. 993.
³ Gerlach und Vogel. Centralbl. f. Bakt. (II) 8: 671. 1902.
⁴ Bredemann. Ibid. 22: 44. 1908; 23: 385. 1909; Idem. Ber. bot. Ges. 26a: 362 and 795. 1908.
⁵ Kostytschew, S., A. Ryskaltschuk und O. Schwezowa. Zeitschr. f. physiol. Chem. 154: 1. 1926.
⁶ Krzemieniewsky. loc. cit.; Stoklosa. Centralbl. f. Bakt. (II) 21: 629. 1908; Omeliansky, W. Arch. d. biol. Wiss. Vol. 18. Part 4. 1914; and others.
⁷ Kostytschew, Ryskaltschuk and Schwezowa. loc. cit.
⁸ Winogradsky, S. loc. cit.

The quantities of fixed nitrogen given in this table are calculated for each 3 grams of sugar fermented. For the various forms of *Bac. amylobacter*, some investigators have observed a N-fixation of 1.4–7.6 mg. per gram of sugar used. The author of this book, by some unpublished experiments, has confirmed the results of Krzemieniewsky concerning the stimulating effect of soil on the fixation of nitrogen by *Azotobacter* but did not obtain analogous results with *Clostridium Pasteurianum*. The highest yields of N were:

For <i>Azotobacter agile</i>	23.3 mg. N per gram of sugar
For <i>Clostridium Pasteurianum</i>	12.5 mg. N per gram of sugar

It is to be noted especially that it does not matter how much sugar is used because the process of N-fixation is a synthesis of ammonia and is therefore *exothermal*.^o It produces 21 calories per mol of NH_3 (in solution). The active hydrogen necessary for the fixation of nitrogen is formed in the processes of respiration and fermentation (*cf.* Chap. VIII).

The chemical side of nitrogen fixation is very interesting but this problem has been attacked only recently. The following sequence has been established for *Azotobacter agile*:¹ the molecular nitrogen is reduced by the bacteria to ammonia which is transferred to amino acids and from these finally made into protein. Under properly selected conditions, ammonia and amino acids can be demonstrated in liquid substrates of *Azotobacter*.

Thus the fixation of nitrogen is a reduction process. The reducing power of *Azotobacter* is surprising; the conditions of N-fixation are comparable to those in the technical operations of Haber-Bosch (p. 146). According to my own experiments (*loc. cit.*), *Azotobacter* also reduces nitrates to NH_3 at an astonishing rate and HNO_2 is formed as an intermediate product.² In the soil the action of *Azotobacter* is thus antagonistic to that of the denitrifying bacteria. Ammonia is probably formed by all the other organisms which fix nitrogen,³ but it was previously supposed that for both *Azotobacter* and *Bacillus radicicola* the first products in the fixation of nitrogen are nitrous acid,⁴ amides,⁵ amino acids⁶ and even hydrogen cyanide.⁷ It is worthy of note that large amounts of urea and the enzyme urease which splits urea, have been demonstrated in the tubercles of the *Leguminosae*.⁸

An estimate of the quantities of molecular nitrogen assimilated in various soils involves a great many technical difficulties and can be made only very

^o This point has been emphasized by Burk (J. Gen. Physiol. 10: 559–573. 1927) for all cases of the fixation of nitrogen, as ammonia or as nitrates. The high consumption of carbohydrates by N-fixers is said to be due to ordinary metabolism.—Ed.

¹ Kostytschew, S. et A. Ryskaltschuk. Compt. rend. 1925; Kostytschew, Ryskaltschuk and Schwewowa. *loc. cit.*; Kostytschew and A. Scheloumow. An unpublished paper.

² Kostytschew, S. und O. Schwewowa. Planta. 2: 529. 1925.

³ Reinke. Ber. bot. Ges. 21: 379. 1903.

⁴ Loew and Aso. Bull. Coll. Agr. Tokyo. 7: 567. 1908.

⁵ Gerlach und Vogel. Centralbl. f. Bakt. (II) 9: 884. 1902.

⁶ Lipman, J. G. Annual Report New Jersey Exp. Sta. 25: 268. 1904.

⁷ Stoklasa, J. Centralbl. f. Bakt. (II) 21: 626. 1908.

⁸ Benjamin. Proc. Roy. Soc. N. S. Wales. 49: 78. 1915.

approximately.¹ In any case it is to be concluded from recent investigations that the importance of the free-living nitrogen-fixing bacteria has so far been underrated.

The favorable effect of legume culture is not due entirely to the assimilation of molecular nitrogen by the tubercles. According to Hiltner's theory,² as the result of their excretions the region about the roots of seed-plants forms a special "Rhizosphäre," i.e., a sphere of local effect of roots on the microflora of the soil. The specific action of the *Leguminosae* is to retard the nitrification in the soil and to increase both the assimilation of nitrogen compounds and the fixation of nitrogen by soil organisms. As the result of the effect, the soil is considerably enriched in nitrogen but a large part of it is not fixed by the *Leguminosae* themselves but by the free-living microorganisms; for example, *Azotobacter* grows particularly abundantly between the roots of the *Leguminosae*.³ According to some unpublished work of the author of this book, but in line with the very meritorious results of Bezsonoff,⁴ it is to be concluded that the N-fixing bacteria use the excretions of the roots of some seed-plants and thereby fix so much nitrogen that a normal crop of those particular seed-plants is assured. According to the latest growth experiments of Scheloumow, *Azotobacter* helps considerably in the growth of tobacco and maize when the soil reaction is alkaline, and especially during the second year after the inoculation.

Therefore the assimilation of nitrogen from the atmosphere is due much more to the activity of the free-living microorganisms than was previously supposed. In southern regions there is a still greater assimilation of atmospheric nitrogen by the soil.⁵ Although there are unfortunately not enough data on this point, the fact is that rich harvests are gathered annually in India without the slightest use of fertilizer even on the worst soil. The cultivated soils in southern lands always require less nitrogenous fertilizers from year to year even for continuous cultivation.⁶ It is quite possible that an accurate investigation of this question will confirm the correctness of the view which is now very probable—that the amount of fixed nitrogen on the earth's surface is now being increased rapidly. At our latitudes the activity of nitrogen-fixing bacteria is suppressed, probably by low soil temperatures and insufficient excretions by the roots of seed-plants. On this account it is absolutely necessary to add manure, green fertilizers and similiar substances to the fields, as the French agricultural chemists correctly emphasized many years ago.⁷ It is not sufficient

¹ Schloesing et Laurent. Ann. Inst. Pasteur. 6: 830. 1892; Berthelot. Compt. rend. 101: 775. 1885; Henry. Jour. d'agr. prat. 61: 411 and 485. 1897 II; 1897 I., P. 549; Kühn. Fühlings landwirt. Zeit. for 1901, p. 2 calculates that free-living soil bacteria in a well worked soil assimilate 66 kg. of nitrogen per hectare annually.

² Hiltner. Arb. d. deutsch. landwirt. Ges. 98: 69. 1904.

³ Beijerinck. Bot. Zeit. 48: 842. 1890; Idem. Verslagen d. Afdeeling Natuurkunde, König. Akad. Wiss., Amsterdam for 1908, p. 53.

⁴ Bezsonoff, N. La science du sol. 1925.

⁵ Leo-Anderlind. Jour. f. Landwirt. 31: 275. 1883; Bergtheil. Agr. Jour. of India. 1: 70. 1906.

⁶ The author of this book observed on the south coast of Crimea growths of tobacco which had produced good crops continually for 35 years on the same land without the use of any fertilizer. The use of saltpeter has no effect on these fields and there is no lack of nitrogen to be found in the soil. This soil proved to be extraordinarily rich in *Azotobacter* (more than 10,000,000 per gram of soil). Cf. Arb. d. Bureau f. landwirt. Microbiologie. Vols. 1 and 2. 1926 and 1927.

⁷ Cf., e.g., Déhérein. Compt. rend. 125: 283. 1897.

to add plenty of mineral fertilizers. The importance of manure as a direct source of carbonic acid for green plants has already been mentioned (p. 146).

12. METHODS OF ISOLATION AND GROWTH OF MICROÖRGANISMS WHICH HAVE CERTAIN BIOCHEMICAL CHARACTERISTICS

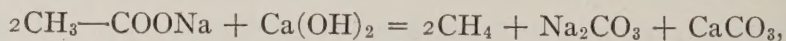
It is impossible to describe here the general methods of isolating pure cultures of micro-organisms as used in microbiology. They are given fully in handbooks of general microbiology.¹ We shall give here only the special methods of isolating and determining the action of the specific bacteria which have been described in this chapter.

The nitrifying bacteria are isolated on inorganic media. Winogradsky and Omeliansky² used the following solutions for elective cultures: To 1 liter of water add 2 g. $(\text{NH}_4)_2\text{SO}_4$, 1 g. K_2HPO_4 , 0.5 g. MgSO_4 , 2 g. NaCl , 0.4 g. FeSO_4 and an excess of basic magnesium carbonate. The following solution was prepared for the nitrate formers: To 1 liter of water add 1 g. NaNO_2 , 0.5 g. K_2HPO_4 , 0.3 g. MgSO_4 , 0.5 g. NaCl and 0.5 g. Na_2CO_3 . Not too little soil should be used for inoculation. For the isolation of pure cultures, inoculation is made from an elective culture to a plate of silicic acid jelly which is saturated with this mineral solution,³ since neither gelatine nor agar suit the purpose. On the contrary, the other microörganisms can be isolated by the ordinary methods of pure cultures so that elective cultures are used only for these bacteria.

To prepare an elective culture of the colorless sulphur bacteria, Winogradsky recommends the use of a piece of the rhizome of *Butomus umbellatus* in a tall glass cylinder filled to the rim with water. To it is added some mud and then a little gypsum. Various sulphur bacteria develop in large quantities under these conditions after one month. To isolate the purple bacteria, an egg is allowed to rot in a tall glass cylinder of water, in the presence of gypsum and in direct light. The purple bacteria collect in a continuous layer on the side of the vessel towards the light. Beijerinck⁴ recommends that a vessel be filled with filthy water containing hydrogen sulphide, and so covered with a glass plate that there is no air space between the glass and the liquid. The plate is to be illuminated only on one side. The purple bacteria adhere to the under side of the lighted plate and can be removed with it.

An elective culture of iron bacteria is obtained thus. Some bog water is mixed with ferrous ammonium citrate. After some time the liquid becomes filled with flocks of *Crenothrix* and *Cladothrix*.

The following solution is used for bacteria which oxidize methane and hydrogen: To each 100 cc. of water add 0.05 g. K_2HPO_4 , 0.1 g. NH_4Cl , 0.02 g. MgSO_4 and traces of FeCl_3 . To obtain the hydrogen bacteria in particular, substitute 0.2 g. KNO_3 for the ammonium chloride. Inoculations can be made from liquid manure, sewage or soil. A temperature of 30–37° is favorable for methane bacteria while 27–30° is the best for hydrogen bacteria. *Bacillus obigocarpophilus* must be isolated in pure cultures on gypsum or paper plates and the other bacteria on ordinary meat-peptone gelatine.⁵ Methane is prepared from sodium acetate according to the reaction:



and the gas washed with bromine water and potassium hydroxide. The hydrogen is prepared from metallic zinc and dilute sulphuric acid and is washed with permanganate, sulphuric acid and concentrated potassium hydroxide. In addition, some CO_2 is added to the culture vessel to provide for the carbon nutrition.⁶

¹ Omeliansky. Principles of microbiology. 4th ed. 1922 (Russian); Abel. Bakteriologie. 1911; Besson. Technique microbiologie. 5th ed. 1911; Omeliansky. Practical introduction to microbiology. 1923 (Russian); [or any of the later books in English such as those by Lutman, Marshall, Giltner, and many others.]

² Winogradsky und Omeliansky. Centralbl. f. Bakt. (II) 5: 333 and 432. 1899.

³ Omeliansky. *Ibid.* 5: 652. 1899; 8: 785. 1902.

⁴ Beijerinck. *Ibid.* 14: 843. 1893.

⁵ For the isolation and culture of the hydrogen bacteria cf. Grohmann, G. *Ibid.* 61: 251. 1924.

⁶ It is even better to grow the culture in an atmosphere of electrolytically prepared oxy-hydrogen gas.

A strong denitrification, which is carried on in rich elective culture, develops from the inoculation of a dilute solution of saltpeter and calcium tartrate or citrate with fertile soil or liquid manure. Some one species of bacteria is preponderant, depending on the organic acid used and the kind of inoculation.

The tubercle bacteria can be isolated on various solid media but best on agar such as the following preparation: 1.5% agar, 0.2% extract of the roots of the plant from which the bacteria are to be isolated, 1% glucose and some CaCO_3 . After heating in an autoclave at 120° for 20 minutes, the liquid is filtered. Diluted human urine can be successfully used to obtain bacteroids. The solutions must always contain large amounts of carbohydrates. The following nutrient solution is recommended for experimental investigations with the tubercle bacteria: 3% grape sugar and 0.1% NaNO_3 in the soil extract or 3% sugar, 1% NaCl and traces of NaHCO_3 in a bean extract.¹

For elective cultures of *Clostridium Pasteurianum*,² Winogradsky used the following solution: To 100 cc. of water add 2–4 g. glucose, 0.1 g. K_2HPO_4 , 0.05 g. MgSO_4 , traces of NaCl , FeSO_4 and MnSO_4 and an excess of CaCO_3 . For inoculation use a soil previously heated to 80° to kill the forms which do not form spores. The culture must contain no oxygen. For each reinoculation, use freshly heated material.

Cultures of *Azotobacter* do well in the following solution: 1 liter of water, 0.2 g. K_2HPO_4 , 0.2 g. MgSO_4 , 0.2 g. NaCl , 0.1 g. CaSO_4 , 5 g. CaCO_3 , 20 g. mannite and 20 g. agar. To regenerate in the bacteria the lost power to fix molecular nitrogen, use a culture in soil or soil extract. In the first case, dried and finely sifted soil from around beets is uniformly moistened with water, then mixed with glucose-agar and sterilized for three-quarters of an hour in the autoclave at 150° .³ The soil extract is prepared as follows: 1 kg. soil is heated with 1 liter water for half an hour at 120° in the autoclave or with 2 liters of water for 2 hours over an open flame. About 600 cc. of extract is thus obtained. This is mixed with talc and passed through a hard filter which leaves the filtrate entirely clear. In a recent communication, Winogradsky⁴ describes an experiment to compare the bacterial strengths of different soils. Pure cultures are not prepared and the soil is mixed directly with a substance like sugar or mannite that will provide energy. A nutrient medium poor in organic compounds or, best of all, silicic acid jelly is said to serve for elective cultures. For determining the bacterial count per unit of soil, Winogradsky recommends giving up poured plates and changing to the use of direct calculations according to the corresponding color of the soil. The comparative nitrifying and nitrogen-fixing power of different soils is measured in the laboratory of the author of this book by the following methods:

*Nitrifying Power of Soil (Waksman's method).*⁵—To 50 g. of sifted soil in a dish is added 0.07 g. $(\text{NH}_4)_2\text{SO}_4$ or (for acid soils) 0.3 g. of lupine meal; the water content of the soil must be 50% of its capacity for water. Several dishes are left at 24° for 14 to 20 days and the nitric acid content is then determined.

*Nitrogen-fixing Power of Soil (Winogradsky's method).*⁶—The work of *Azotobacter* is measured as follows. Large Petri dishes of silicic acid jelly, mineral salts and 2 g. of mannite to each 200 cc. of jelly are mixed with finely sifted soil (0.5 g. to each dish); fixed nitrogen must be excluded. The colonies of *Azotobacter* are counted after 6 days. To demonstrate the activity of these bacteria, the amount of fixed nitrogen must be determined. For the direct determination of nitrogen it is better to use soil mixed with mannite and pressed firmly into Petri dishes.⁷

According to Winogradsky, the work of *Clostridium Pasteurianum* is measured in the same way except that the mannite is replaced by grape sugar and the dishes are left in an atmosphere of pure nitrogen.

¹ Mazé, P. Ann. Inst. Pasteur. 11: 45. 1897; 12: 1. 1898.

² Winogradsky, S. Arch. d. biol. Wiss. 3: 297. 1897 (Russian).

³ Bredemann. Ber. bot. Ges. 26a: 366. 1908.

⁴ Winogradsky, S. Ann. Inst. Pasteur. Vol. 39. 1925.

⁵ Waksman, S. Soil Science. Vol. 25. 1923.

⁶ Winogradsky, S. Compt. rend. 180: 711. 1925; Ann. Inst. Pasteur. 40: 455. 1926; 42: 36. 1928; Compt. rend. du 7 Congrès de Chim. Ind. 1927.

⁷ loc. cit.

CHAPTER IV

PLANT NUTRITION WITH PREPARED ORGANIC COMPOUNDS

I. THE CARBON AND NITROGEN NUTRITION OF MICROÖRGANISMS FREE FROM CHLOROPHYLL

With the exception of the microörganisms capable of chemosynthesis (see previous chapter), all the other chlorophyll-free plants are forced to feed on prepared organic compounds. It would be premature to claim (as it has been done in popular writings) that the nutrition of chlorophyll-free plants is fundamentally the same as that of animals. In by far the greater number of cases the plant without chlorophyll is nourished only with prepared organic substances that are free from nitrogen and it synthesizes nitrogen compounds from inorganic salts of nitrogen. The animal is not capable of this synthesis; it has been proved that it cannot synthesize many amino acids at the expense of ammonia, even when the carbon skeleton of each amino acid is placed at its disposal in the form of the corresponding ketone acid.¹ An animal must take in foods both with and without nitrogen and only in the form of prepared organic compounds, *i.e.*, carbohydrates and proteins, or at least as the structural units of the protein molecule—the amino acids. The nutrition of seed-plants without chlorophyll has been studied very little. Lately they have been considered in connection with the problem of parasitism. The nutrition of the lower, non-green plants—bacteria and fungi—has been studied in more detail because these organisms are easily grown in pure cultures on solutions of definite composition. Then by the use of exact chemical analyses, the balance of each food material and all the products of physiological changes can be measured.

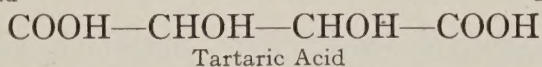
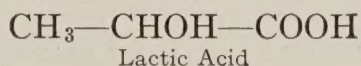
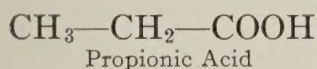
Two well-marked physiological groups of microörganisms without chlorophyll can be separated from each other. The first group—that of strict specialists—has been described in part in the preceding chapter, and in part will be explained in the consideration of fermentations. There are these organisms which bring about definite chemical transformations of organic substances which are peculiar to no other plants. The chemical specificity in some respects permits them to remain apart from the general “struggle for life.” They actually use substances which belong in the category of waste-products of other plants or they have the capacity to exist under severe conditions which would not support the life of the majority of the higher or lower plants. Hence the strict specialists find proportionately less competitors. It was mentioned above that these microörganisms also play prominent rôles in completing the cycles of various physiologically important substances.

¹ Abderhalden, E. *Zeitschr. f. physiol. Chem.* 96: 1. 1915.

The other category—that of the omnivorous microorganisms—stand the battle for existence thanks to their modest needs and their ability to feed on various kinds of organic substances and even on very poor food materials. Here belong most of the so-called saprophytic fungi and bacteria. They all play an important geochemical rôle in that they decompose animal and plant remains, form humus and peat and also bring about the mineralizing of these products.¹ This process offers considerable interest but a more detailed explanation must be left to the text books of soil science and microbiology; it meets our purposes to explain the questions of general physiological significance.

From the foregoing statement it is clear that there would be no chance of putting together a nutrient solution which would provide for a good growth of all microorganisms. In a series of papers 50 years ago, Pasteur² developed the idea of the specificity of the nutrition of bacteria. In spite of this, different investigators have tried to prove a certain sequence of nutritive values of the various organic substances. At best, this would be feasible only for single specific organisms but there has been a whole series of controversies in this limited field. It is quite unnecessary to cite the various “sequences of nutritive values” which have been published by different writers, because they are of historical interest only. The fact is that every series of this kind changes according to the external conditions and the stage of development of the microorganism,³ the temperature,⁴ the nitrogen nutrition⁵ and other factors are all important. The one fact common to all the sequences is that sugar proves to be the best source of carbon for the omnivorous microorganisms and especially for the fungi.

The non-sugars do not act alike in different cases and we can at most only point out some general relationships. For example, substances with a straight carbon chain are more easily assimilated than their isomers with branched chains; the lowest members of a homologous series are assimilated less than the compounds with three, four or more carbon atoms; substances with several hydroxyl groups are much better sources of carbon than substances with unoxidized carbon atoms. Thus lactic acid is a better source of carbon for fungi than propionic acid,⁶ and tartaric acid is better than lactic acid which contains one methyl group CH₃:⁷



A general rule for the nutrition of the omnivorous fungi has been recently proved by experiments. It was found that carbohydrates and proteins are the

¹ Winogradsky, S. *loc. cit.* describes various microbes which bleach and break down soil humus.

² Pasteur, L. *Compt. rend.* 45: 2 and 913. 1857; 47: 224. 1858; 48: 337 and 640. 1859; *Idem.* *Ann. de chim. et de physique* (3) 52: 404. 1858; 58: 323. 1860; *Idem.* *Etudes sur le vinaigre.* 1868; *Idem.* *Etudes sur la bière.* 1876; and many others.

³ Duclaux. *Ann. Inst. Pasteur.* 3: 67. 1889.

⁴ Thiele, R. *Die Temperaturgrenzen der Schimmelpilze.* Diss. Leipzig. 1896.

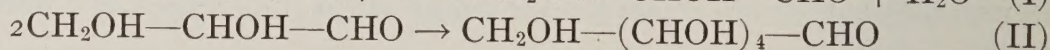
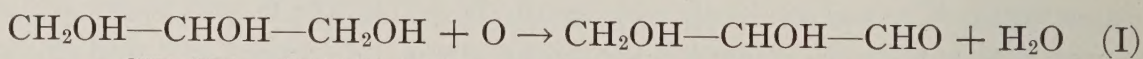
⁵ Ritter, G. *Materials for the physiology of the molds.* 1916 (Russian).

⁶ Troili-Peterson. *Centralbl. f. Bakt.* (II) 24: 333. 1909.

⁷ Kostytschew, S. and M. Afanassjewa. *Jour. russ. bot. Ges.* 2: 77. 1917 (Russian).

primary substances for fungi as well as for green plants. Such nitrogen-free, organic substances as mannite, glycerine, tartaric acid, lactic acid and quinic acid are changed by the fungi to sugar, usually to glucose.¹ This was proved by the method of artificial interference in the biochemical process (p. 62). Under normal living conditions the sugar formed by the fungus from various organic substances is at once used for its vital requirements; but if an aerobic fungus has its supply of air disturbed, the part of the metabolism which involves the consumption of sugar is retarded and the sugar formed from various non-sugars accumulates in the substrate.

Consequently the unequal nutritive value of the various nitrogen-free, organic compounds for the omnivorous plants is based on the unequal ease with which they form sugar from these substances under different conditions. This result agrees with the empirically-established, relative nutritive values of the various substances without nitrogen; a compound with a straight carbon chain and several hydroxyl groups can change to sugar more easily than another substance which can be transformed into sugar only by a complicated synthesis, particularly if it is thereby necessary to oxidize several carbon atoms. Hexavalent alcohols, such as mannite and sorbite, and even pentavalent alcohols with six carbon atoms, like quercite, are excellent foods for molds² because the transformation of these alcohols into sugar involves only an oxidation. The trivalent glycerine, which is easily changed to sugar



is likewise an excellent source of carbon (only when fungi are fed with unoxidized nitrogen!) but, on the contrary, the lower alcohols, which are much less suited to the synthesis of sugar, have a low nutritive value especially if their carbon atoms are not all oxidized. The relative nutritive values of alcohols for the fungus *Aspergillus niger* is illustrated by the following table, taken from the paper by Czapek.³ The figures indicate the dry weight of the fungus crop in milligrams.

Ethyl alcohol.....	0 mg.	d-Mannite.....	416.1 mg.
Ethylene glycol.....	74.3 mg.	d-Sorbitol.....	542.5 mg.
Glycerine.....	288.6 mg.	Dulcitol.....	27.3 mg. (!)
Erythritol.....	323.8 mg.	d-Fructose.....	523.7 mg.

The hydroxy acids, particularly malic, citric, tartaric, lactic, glyceric and fumaric acids,⁴ are very good sources of carbon, *i.e.*, good material for the building of sugar. Acetic, propionic, formic and succinic acid are harder to assimilate.⁵ There is the same obvious connection with the synthesis of sugar here as in the use of alcohols for food.

¹ Kostytschew, S. Zeitschr. f. physiol. Chem. 111: 236. 1920; Kostytschew und Afanassjew. Jahrb. wiss. Bot. 60: 628. 1921.

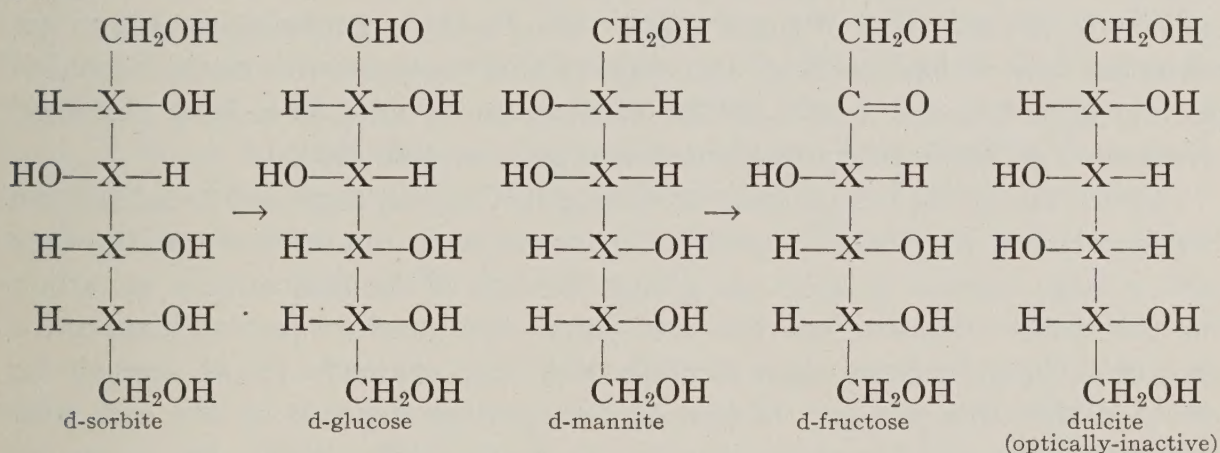
² Czapek, F. Hofmeisters Beitr. 1: 538. 1902.

³ Idem. Hofmeisters Beitr. 3: 62. 1902.

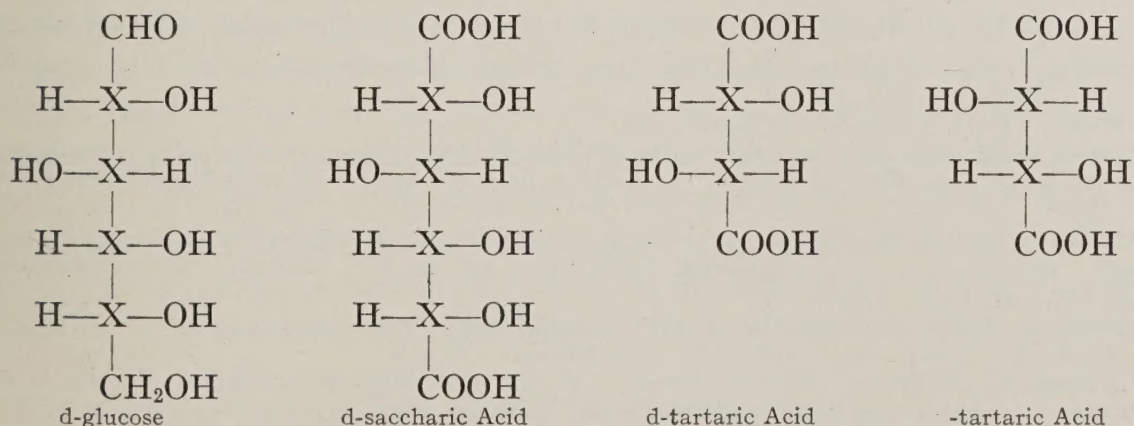
⁴ Schukow. Centralbl. f. Bakt. (II) 2: 601. 1896; Nägeli, C. Bot. Mitt. 3: 395. 1879; Pfeffer. Pflanzenphysiologie 1: 372. 1897.

⁵ Pfeffer. *loc. cit.*; Salzmann. Centralbl. f. Bakt. (II) 8: 349. 1902.

In connection with the ease with which sugars are synthesized, we have the unequal nutritive values of the stereoisomeric alcohols and hydroxy acids, already pointed out in Chap. I. In judging the results, it must be borne in mind that the sugars serve chiefly as material for the process of respiration which releases energy. This material could only (*cf.* Chap. VIII) be sugars which are capable of alcoholic fermentation and these are d-glucose, d-mannose, d-fructose and d-galactose. Consequently these sugars must be made from non-sugars. In the foregoing table of relative nutritive values of various alcohols, the low value of dulcitol is striking in comparison with those of the stereoisomeric mannitol and sorbitol. If the projection formulae are taken into consideration, it is at once evident that sorbitol changes directly to glucose and fructose by the oxidation of one carbon atom while mannitol changes to fructose or mannose, but dulcitol is optically inactive on account of its symmetrical structure and cannot be used directly as material for the making of fermentable sugars. On the contrary, it is necessary to rebuild the dulcitol molecule and so it is assimilated by the fungus even more poorly than the first member of the series, ethylene glycol.



The dextra-rotatory tartaric acid, which occurs only in plants, is a good food for fungi while the levo-rotatory tartaric acid is either not utilized at all or only very little by the microorganisms, with few exceptions.¹ Now it is known that between glucose and dextra-rotatory tartaric acid there is a direct connection by way of saccharic acid² while the levo-rotatory tartaric acid has another spatial arrangement of atoms.



¹ Pasteur, L. Compt. rend. 46: 617. 1858; 51: 298. 1860.

² Fischer, E. Ber. chem. Ges. 29: 1377. 1896.

A well known and very characteristic example of the same kind is the unequal assimilation of fumaric and maleic acid by microörganisms. Fumaric acid is a good source of carbon but maleic acid cannot be used.¹ The former occurs in plants and is closely related to tartaric and malic acid; the latter has never been found in plants. In the first chapter, other cases of the selectivity of plants for stereoisomeric substances were discussed; only such facts will be mentioned here as have a direct relation to the biological synthesis of sugars.

The proof of the synthesis of sugar in the nutrition of omnivorous organisms at the expense of non-sugars explains the interesting observation that in the presence of two different sources of carbon, the molds are nourished only with the one which has the highest nutritive value and the other is left untouched.² Of the reactions which lead to the synthesis of sugar, there take place only those which involve the least expenditure of energy.

Up to this point we have considered only the good sources of carbon which provide for a luxuriant growth. The unassuming omnivorous organisms are still able to develop at the expense of very poor sources of carbon, which are usually classed as waste-products. These are oftentimes very pronounced poisons to the nutrition of some fungi. Thus many organisms are able to get along not only with acetic acid³ and ethyl alcohol⁴ but also with methyl alcohol,⁵ acetaldehyde,⁶ formic acid⁷ and like substances. Plants have been described which feed on aliphatic hydrocarbons and grow on paraffin oil.⁸

Under the cyclic compounds, according to Czapek, come substances of the benzene series, in general a poor nutrient material, but hydroaromatic rings with a large number of hydroxyl groups are one of the best sources of carbon for the molds. Inosite, quercite and quinic acid produce just as luxuriant a growth of fungi as hexavalent alcohols with open chains.⁹ It can scarcely be doubted that this property of hydroaromatic rings depends on the ease with which they break, after which there are no further difficulties in the formation of the sugar molecules. The various kinds of glucosides are split just as easily by fungi and bacteria, but most of the sugar contained in the glucoside is thereby used up.

The method of comparative studies of the nutritive values of various sources of carbon for the fungi consists in determining first, the time required to form a fungus mat that hangs together; second, the maximum dry weight of the fungus which grows in a certain amount of liquid; third, the so-called economic coefficient or the ratio of the dry weight of the fungus crop

¹ Buchner. Ber. chem. Ges. 25: 1161. 1892; Czapek. Hofmeisters Beitr. 2: 584. 1902; Dox. Jour. Biol. Chem. 8: 265. 1910.

² Pfeffer. Jahrb. wiss. Bot. 28: 205. 1895.

³ Hasselbring. Bot. Gaz. 45: 176. 1908; Molisch, H. Sitzungsber. d. Akad. Wien. Math.-natur. Kl. I 103: 562. 1894; Will und Leberle. Centralbl. f. Bakt. (II) 28: 1. 1910; Raciborski. Flora. 82: 115. 1896.

⁴ Mazé. Ann. Inst. Pasteur. 18: 277 and 378. 1904; Will, H. Centralbl. f. Bakt. (II) 34: 9. 1912; Schnell. Ibid. 35: 24. 1912; Lindner, K. und Cziser. Ber. bot. Ges. 29: 403. 1911; Stockhausen. Chem. Zeit. 35: 1197. 1912.

⁵ Loew, O. Centralbl. f. Bakt. (II) 12: 462. 1892; Bokorny. Ibid. 29: 176. 1911.

⁶ Perrier, A. Compt. rend. 151: 163. 1910; Bokorny. loc. cit.

⁷ Franzen, H. und Braun. Biochem. Zeitschr. 8: 29. 1908; Franzen und Greve. Zeitschr. f. physiol. Chem. 64: 169. 1909; Franzen und Steppuhn. Ibid. 77: 129. 1912; 83: 226. 1913.

⁸ Rohn, O. Centralbl. f. Bakt. (II) 16: 382. 1906; Gola. Bull. Soc. Bot. Ital. Ottobre 1912.

⁹ Nägeli, C. Bot. Mitt. 3: 395. 1879; Czapek. Hofmeisters Beitr. 1: 538. 1902; 2: 557. 1902.

to the amount of food material used. The larger the economic coefficient, the higher is the value of the source of carbon being studied. That is, a high coefficient shows that only an insignificant part of the food material is used for processes other than growth and reproduction. Under optimum conditions for growth, this coefficient for the fungus *Aspergillus niger* equals $\frac{1}{3}$ when fed on sugar; a higher value has never been obtained.

The chemical side of the transformation of various substances into sugar was not studied until recently. The method of artificial interference in the biochemical process should make it possible to isolate the intermediate products of the change of various non-sugars to sugar, and consequently to clear up the chemistry of this process. Some orienting points in this direction have already been obtained.¹

As for the nitrogen nutrition of plants without chlorophyll, it must be emphasized that it is not like that of animals. In the first place, almost all plants are able to feed on inorganic nitrogen compounds. This was proved for the fungi by the classical work of Pasteur and his students. With respect to the bacteria, it has been proved recently that the majority of them can be grown in the absence of complicated compounds of nitrogen. One of the widely used nutrient solutions—that of Uschinski—has the following composition: To 1 liter of water add 30–40 g. glycerine, 5–7 g. NaCl, 0.1 g. CaCl₂, 0.4 g. MgSO₄, 3 g. K₂HPO₄, 6–7 g. ammonium lactate and 3–4 g. asparagine.² It must be borne in mind that nutrition with a single amino compound, such as asparagine, is due to nutrition with ammonia, since in the case before us a synthesis of all essential amino acids and hence of the protein molecule takes place only after deaminization of asparagine.

Secondly, the nitrogen nutrition of fungi and bacteria approaches that of green plants in the sense that in neither fungi and bacteria nor green plants is there any loss of nitrogen during the vital metabolic changes, whereas the animal excretes daily a considerable amount of nitrogen in the excrement and urine and requires a steady compensation for this loss. All the foregoing characteristic biochemical properties of plants also apply to the fungi and bacteria.

On the contrary, if we consider the importance of the various sources of nitrogen for plants without chlorophyll *from the quantitative side*, it becomes evident that there are unequal nutritive values of the various nitrogen compounds for the microorganisms. In general we can find three categories of nitrogenous foods: the first includes the inorganic compounds, ammonia, nitric acid and nitrous acid; the second covers organic substances which serve as sources of nitrogen after mineralization has taken place; and the third includes the organic nitrogenous substances which are used in the synthesis of protoplasmic proteins without splitting off inorganic compounds of nitrogen. Under this comes prepared proteins, peptone preparations and mixtures of amino acids which are contained in the proteins of the organism that is being studied.

¹ Kostytschew, S. Zeitschr. f. physiol. Chem. **III**: 236. 1920.

² Uschinsky. Centralbl. f. Bakt. (II) **14**: 316. 1894; cf. also Fränkel, C. Hyg. Rundschau. **4**: 769. 1895; Voges, O. Centralbl. f. Bakt. (II) **15**: 453. 1894; and others.

Among the inorganic compounds, the ammonium salts are by far the best source of nitrogen for fungi which usually tolerate an acid reaction very well; the latter is easily produced by the relatively slow absorption of the anions of the ammonium salts. Some molds, the nutrition of which has been thoroughly studied, such as *Aspergillus niger* and *Penicillium glaucum*, attain their maximum development when fed with ammonia. This development cannot be exceeded even when fed with prepared organic (protein) compounds. Also some bacteria, such as *Bacillus subtilis*, grow best on a nitrogen nutrition at the expense of ammonia.

Nitric acid and nitrous acid are less suitable sources of nitrogen for plants without chlorophyll, in comparison with ammonia. Nitrous acid as a source of nitrogen exceeds ammonia in nutritive value only for the nitrate formers. Among the molds, only a few, including *Cladosporium herbarum*, *Mucor racemosus* and *Mucor spinosus*, are able¹ to grow well on nitrates. Other fungi which are able to use nitrates for their nutrition can also assimilate nitrites but only after reaching a certain stage of development. The initial growth of some fungi either does not take place when fed on nitrites or is very meager. Under the conditions used to grow fungi, the nutritive value of nitrates is always higher than that of nitrites. Some earlier investigators even supposed that nitrates are a better source of nitrogen for some fungi than ammonia: the latest investigations do not confirm this. It turns out that only a few fungi, such as *Mucor racemosus*, *Cladosporium herbarum* and *Aspergillus glaucus*, assimilate nitrates as well as ammonium salts when fed with sugar.² Other fungi, like *Rhizopus nigricans*, *Mucor Mucedo* and *Thamnidium elegans*, are entirely unable to use oxidized nitrogen, while a third group, including *Aspergillus niger*, *Penicillium glaucum* and *Botrytis cinerea*, occupy a middle position.³ They are able to get along on nitrates but prefer ammonia.⁴

When the sugar is replaced by other sources of carbon, the nitrates can be assimilated less easily so that the difference between nitrate and ammonia feeding becomes very distinct. The fungi of the first category, which assimilate nitrates almost as well as ammonia when fed on sugar, clearly prefer the latter when growing at the expense of mannite or glycerine. Fungi of the type of *Aspergillus niger* usually cannot feed on nitrates if glycerine is the source of their carbon. In this case free nitric acid accumulates in the substrate if the nitrogen is offered in the form of ammonium nitrate.

In the case of feeding with nitrogenous organic compounds not of protein nature, the fungi and bacteria first remove ammonia or nitric acid. Since the synthesis of protein represents the end result of the assimilation of nitrogen, wherefore all the amino acids contained in the protein molecule must first be formed, it is self-evident that a direct synthesis of protein from a simpler organic compound is impossible if the compound itself is one structural unit of the protein molecule. This question will be treated fully in considering the physio-

¹ Ritter, G. Ber. bot. Ges. 29: 570. 1911.

² Laurent. Ann. Inst. Pasteur. 2: 593. 1888; 3: 362. 1880.

³ Ritter, G. Materials for the physiology of the molds. P. 1-43. 1916 (Russian).

⁴ Ritter, G. loc. cit. It is interesting that, according to Ritter, molds can assimilate free nitric acid.

logical rôles of asparagine and glutamine in the seed-plants. If for many microorganisms and particularly bacteria, the organic nitrogenous substances under certain conditions have a higher nutritive value than ammonia, the explanation must lie in secondary effects. In the assimilation of ammonia there accumulates in the substrate the free acid which formed a part of the ammonium salt. This gives an acid reaction to the nutrient medium and the effect is unfavorable especially on the bacteria but also on some molds. For the same reason the reaction of the nutrient solution becomes alkaline when nitrate ions are assimilated. All salts whose ions are assimilated by the organism with unequal velocities are called physiologically acid or alkaline according to the reaction which is left when the salt is assimilated. Now the assimilation of nitrogen from organic substances does not involve a change of reaction, or at least it produces a less marked change than the feeding of mineral compounds of nitrogen, since the carbon framework of nitrogenous organic compounds cannot be used by plants. Moreover, ammonium salts in appreciable concentrations are toxic for many microorganisms, but they do not accumulate when ammonia is gradually split off from an amino acid. Thus an organic nitrogen compound can be a source of both nitrogen and carbon at the same time. However, it has been proved by experiments that microorganisms generally do better if there is a good source of carbon, especially sugar¹ contained in the substrate, in addition to the organic nitrogen. On account of the importance of carbon nutrition with non-sugars, this condition can be understood as a sugar-forming process and requires no further explanation. Single amino acids and their amides, such as asparagine, are an excellent, and in practice, a much used source of nitrogen for all microorganisms. Various bacteria and fungi, however, can also assimilate the nitrogen of simple amines,² alkaloids,³ nitro compounds⁴ and even derivatives of cyanogen.⁵ Really there are scarcely any nitrogen compounds which cannot serve as sources of nitrogen for some group of microorganisms; even such substances as hydrazine, thiocyno acid and oxime⁶ are assimilated.

With respect to the sources of nitrogen, the same quantitative rules can be found as those for sources of carbon. Just as the relative nutritive values of substances without nitrogen are limited by the ease of their transformation into sugar, so the decisive factor here is the ease with which ammonia is formed from the various substances which contain nitrogen. The quality of the carbon nutrition is also important; the degree of assimilation of the various sources of nitrogen depends to a large extent on the available energy which is used in rearranging the substances containing nitrogen and provided when the nitrogen-free material is oxidized in respiration. Suggestions along this line have already been given in the discussion of nitrate assimilation.

¹ Czapek. Hofmeisters Beitr. 1: 538. 1902; 2: 557. 1902; 3: 47. 1902.

² Demoussy, E. Compt. rend. 126: 253. 1898.

³ Lutz, L. Bull. Soc. Bot. France. 50: 118. 1903; Czapek. *loc. cit.*

⁴ Bokorny. Chem. Zeit. 20: 985. 1896; Czapek. *loc. cit.*

⁵ Reinke. Untersuch. d. bot. Lab. Univ. Göttingen. 3: 37. 1888; Fermi. Centralbl. f. Bakt. 15: 722. 1894; Lutz, L. Compt. rend. 140: 665. 1905.

⁶ Fernbach. Compt. rend. 135: 51. 1902; Kastle, J. H. and E. Elvove. Amer. Chem. Jour. 31: 550. 1904.

Spatial isomerism is also important, particularly for the amino acids. This point was explained for the nitrogen-free food substances and it is sufficient here to notice that there is in living organisms a specific set of enzymes for the working over and deaminization of the amino acids which are contained in the protein molecule; for this reason the spatial isomers of amino acids which occur in the organism are naturally preferred. Thus if an optically inactive *dl*-amino acid is placed at the disposal of the fungi, the isomer which does not occur in the proteins of animals and plants will be left untouched.¹

This type of nutrition with organic nitrogenous substances differs, from the biochemical standpoint, from those cases in which the protein molecules are synthesized entirely or to a large extent without the removal of NH_3 from nutrient material, because the necessary structural units for the synthesis of the protein molecule are offered to the organism ready-made. As mentioned above, this is the only type of nitrogen nutrition that is similar to that of the animals. The outstanding investigations of Abderhalden² showed that the animal can put together proteins from amino acids but is unable to synthesize most amino acids. Among the plants without chlorophyll, we find an exception only in the group of parasites (*diphtheria bacillus*, etc.) the nutrition of which is not entirely normal. However, there are quite a few microorganisms which definitely prefer organic nitrogen foods to mineral nutrition and develop slowly and poorly when fed with ammonia.

Although Pasteur³ proved long ago by his epoch-making work that yeast plants in pure cultures get along with ammonia, the fact is to be emphasized that yeast grows much better on peptone or on a mixture of different amino acids. This also characterizes the bacteria of lactic acid fermentation and apparently to a greater or lesser degree other organisms of fermentations. Thus *Monilia* grows much more poorly when fed with only one amino acid (*i.e.*, *de facto* with inorganic nitrogen) than with peptone.⁴ Of course when fed peptone and protein-like substances, there is not a proportionate use of the single amino acids, because the proportion of the amino acids in the proteins of the protoplasm is not the same for different microorganisms, and it does not correspond in every case to the ratio of these substances in the preparations of peptone, meat extracts and other analogous sources of nitrogen.

In regard to the chemical side of the assimilation of nitrogen by plants without chlorophyll, only the reduction and assimilation of nitrates and nitrites will be considered here because the synthesis of protein from ammonia and amino acids will be treated fully in Chap. VI. Nitrites have been found in the nitrate nutrition of microorganisms just as in seed-plants. In these cases, however, the formation of nitrites could be definitely proved, thanks to the better methods available. By using the method of interference in the biochemical process,

¹Schulze, E. und Boshard. Zeitschr. f. physiol. Chem. 10: 134. 1886; Emmerling, O. Ber. chem. Ges. 35: 2289. 1902; Ehrlich, F. Biochem. Zeitschr. 1: 8. 1906; 8: 438. 1908; Idem. Ber. chem. Ges. 40: 2538. 1907.

²Abderhalden, E. Zeitschr. f. physiol. Chem. 96: 1. 1915.

³Pasteur, L. Ann. de chim. et de physique. 58: 323. 1860.

⁴Went. Jahrb. wiss. Bot. 36: 611. 1901.

it has been possible to show that there is no accumulation of nitrites under normal conditions because the rate of consumption of nitrites exceeds that of the formation of nitrates. The ratio of the velocities of formation and use of HNO_2 is changed by retarding the synthetic processes and the unused nitrites accumulate in the substrate. This can be done for the molds either by producing an alkaline reaction of the substrate, which is generally unfavorable for fungi¹ or by cutting off the supply of sugar, the source of energy, or finally, for the aerobic fungi, by removing the oxygen.² It has also been proved for the molds that nitrites are reduced to ammonia and that the ammonia is changed to the amino group. All these transformation products of oxidized nitrogen can be demonstrated in the liquid substrate of fungi. The reduction of nitrites to ammonia and the synthesis of amino acids takes place only in the presence of considerable amounts of sugar (on the contrary, it is possible to reduce nitrates to nitrites in the absence of sugars). This shows that nitrates and nitrites are assimilated by molds by way of complete reduction to ammonia. It was explained above that the same mechanism of assimilation of oxidized nitrogen has been found for the single-celled green alga *Chlorella*.

In summary we may say that the nutrition of chlorophyll-free plants with organic substances amounts to synthesis of carbohydrates and proteins, just as in the green plants. However, the conditions for these processes are very unlike. Combinations of various sources of carbon with different sources of nitrogen fail to give equally good results. The influence of external factors also modifies the assimilation of various substances with and without nitrogen. For each microorganism there is an optimum temperature at which it develops better than at higher or lower temperatures. Likewise there is a maximum and a minimum temperature and development is possible only within this range of temperatures.³ The assimilation of various substances also depends largely on their concentration;⁴ for each substance there is an optimum concentration which is often very low as it proves to be with nitrites for example. An accurate comparison of the nutritive values of different substances can be made only by considering optimum concentrations, a point which has been entirely disregarded in the past. The age of the fungus is very important for the assimilation of some substances. For example, nitrites are utilized by the mature but not by young cultures of some fungi.⁵ Other external conditions affect the carbon and nitrogen nutrition of plants without chlorophyll; the pH of the medium is very important. After all, as it was explained in Chap. II, it must be borne in mind that the so-called optimum is a complicated phenomenon.

2. THE NUTRITION OF GREEN PLANTS WITH ORGANIC SUBSTANCES

In general, experiments in feeding seed-plants with prepared organic compounds, either with or without nitrogen, have led to the conclusion that this

¹ Ritter, G. Materials for the physiology of the molds. P. 46. 1916 (Russian).

² Kostytschew, S. und E. Tswetkova. Zeitschr. f. physiol. Chem. **111**: 171. 1920.

³ Thiele. Temperaturgrenzen der Schimmelpilze. Diss. Leipzig. 1896.

⁴ Ritter, G. *loc. cit.*; cf. also Pringsheim. Zeitschr. f. Bot. **6**: 577. 1914; Artari. On the physiology and biology of the Chlamydomonads. 1913 (Russian).

⁵ Kostytschew, S. and Tswetkova. *loc. cit.*

nutrition is inferior to the normal one but this result probably depends largely on the accessory circumstances. The main point is that the roots of seed-plants are not adapted to the absorption of organic foods with high molecular weights. The feeding of severed leaves which are floated on the surface of an aqueous solution of various substances, gives better results. Green algae absorb organic substances just as well.

The method of pure cultures of seed-plants has now been developed so far that it is possible to raise seed-plants in darkness for their whole period of growth on solutions of sugar and related substances.¹ Boehm² was the first to prove the possibility of absorption of sugar by roots. After these first experiments, many others were undertaken in darkness as well as in the light.³ Sugar and mannite serve to nourish the green plants but the salts of fatty acids,⁴ ethyl alcohol⁵ and other substances far removed from the carbohydrates are not assimilated by the seed-plants. Thus it appears that the seed-plants are more particular than the microorganisms in relation to organic substances. Amides and amino acids are assimilated easily by the green plants.⁶ Some investigators state that there is no preliminary removal of NH_3 in this process.

In the artificial feeding of leaves, the accumulation of starch has been the chief criterion of nutrition.⁷ Almost all sugars and different polyvalent alcohols, including glycerine, are suited to this purpose. However, organic acids do not function in this respect.⁸ In Palladin's⁹ experiments with artificial feeding of green and etiolated leaves with sugar, there was always a considerable increase in the organic substances and an increase in turgor in comparison with the unfed leaves. A quantitative measurement of the absorption of carbohydrates in the artificial feeding of leaves has also been made.¹⁰ In some cases this amounts to 5 g. per square meter of leaf surface.

It was noticed long ago that light always has a favorable influence on the artificial feeding of seed-plants with carbohydrates.¹¹ In this connection it has been shown recently that peas always absorb the same amount of sugar with different light intensities and that feeding seed-plants with a 4% solution of glucose does not replace the nutrition through light.¹² Now we know that

¹ Petrow, G. G. Assimilation of nitrogen by seed-plants in the light and in darkness. 1917 (Russian).

² Boehm, J. Bot. Zeit. 41: 54. 1883.

³ Laurent, J. Compt. rend. 125: 887. 1897; 127: 786. 1898; 135: 870. 1902; Lutz. Ann. Sci. Nat. (8) 7: 1. 1898; Molliard. Compt. rend. 141: 389. 1905; 142: 49. 1906; Idem. Rev. gén. bot. 19: 242. 1907; Lubimenko. Compt. rend. 143: 130 and 516. 1906; Lefèvre. Ibid. 141: 211, 664, 834 and 1035. 1905; 142: 287. 1906; 143: 322. 1906; Mazé. Ibid. 128: 185. 1899; 139: 470. 1904; Baessler. Landwirt. Versuchs-Sta. 33: 231. 1886; Kawakita, J. Bull. Coll. Agr. Tokyo. 6: 181. 1904.

⁴ Lövinson, O. Bot. Centralbl. 83: 1. 1900.

⁵ Bokorny. Centralbl. f. Bakt. (II) 30: 53. 1911; Mazé et Perrier. Ann. Inst. Pasteur. 18: 721. 1904.

⁶ Lutz. loc. cit.; Baessler. loc. cit.

⁷ Boehm, J. Bot. Zeit. 41: 33. 1883; Meyer, A. Ibid. 44: 81. 1886; Laurent. Bull. Soc. Bot. Belg. 26: 243. 1888; Treboux. Ber. bot. Ges. 27: 438 and 507. 1909; Lindet. Compt. rend. 152: 775. 1911; Nadson. Arb. d. Petersburg Ges. d. Naturforscher. 20: 73. 1889 (Russian).

⁸ Mangin. Compt. rend. 108: 716.

⁹ Palladin, W. Rev. gén. bot. 6: 201. 1894; 8: 225. 1896; 13: 18. 1901; Idem. Ber. bot. Ges. Vol. 20. 1902.

¹⁰ Saposchnikoff, W. Ber. Bot. Ges. 7: 258. 1889.

¹¹ Lefèvre. loc. cit.; Bokorny. loc. cit.; and others.

¹² de Besteiro, Dolores et Michel-Durand, E. Compt. rend. 168: 467. 1919; Idem. Rev. gén. bot. 31: 94. 1919.

plants in the light are nourished chiefly through photosynthesis and prepared organic compounds cannot reduce the rate of the process in leaves. The conclusion is important for agriculture as well as theoretically. In Chap. II there was mention of the humus theory of seed-plant nutrition, which was developed because there was some question whether the low CO_2 content of the atmosphere could meet the needs of plants. In spite of the removal of this uncertainty by the exact work of de Saussure and Boussingault, the humus theory continued to be held and the controversy over it held the attention of the whole learned world during the first half of the past century. Although the principal opponents of the theory—Boussingault and Liebig—gained the upper hand in the battle of theory, the humus theory continued to be held because it was based on farmers' observations. Up until recently there have still been experiments to revive the importance of humus as the direct source of carbon for seed-plants.¹ This concept is now regarded as superceded. For one thing, we are convinced that soil humus has a low nutritive value and, on the other hand, it is found that even excellent food substances do not retard the nutrition of seed-plants through photosynthesis. Finally, there have been described a large number of facts which emphasize the importance of soil humus in the sense of an indirect influence of crops. The adsorptive capacity of the soil for mineral salts increases as humus is added and the assimilation of free nitrogen is also thereby increased (p. 173). Likewise the abundant liberation of CO_2 from humus soils has a favorable action on the photosynthetic nutrition of green plants in the light. In addition some constituents of peat and of humus seem to function as stimulants of growth (Chap. VII).

The artificial feeding of algae with prepared organic compounds often gives better results than in the case of seed-plants. Since we have been able to prepare pure cultures of algae² it has become possible to carry on experiments in the artificial nutrition of algae. It appears that the feeding of organic substances often favors the development of various algae. The *Diatomaceae* show an especially luxuriant growth in the presence of organic compounds and many *Volvocinaceae* also swallow living food; not only bacteria and small *Cyanophyceae* but also diatoms and green algae have been found in them.³ The presence of *Cyanophyceae* and some species of *Spirogyra* in water is a sure indication that there are impurities in that water and serves as a criterion of the quality of it as drinking water. In pure cultures *Nostoc* grows better in the presence of organic substances than in purely mineral solutions.⁴ On the other hand, it is known that *Cyanophyceae* are also able to develop in purely mineral solutions.⁵ Just as for seed-plants, the best foods for algae are the sugars and the polyvalent alcohols related to them.⁶ Still algae are usually unassuming, like seed-plants, because they are able to synthesize carbohydrates

¹ Cailletet. Compt. rend. 152: 1215. 1911; and others.

² Beijerinck. Centralbl. f. Bakt. 13: 368. 1893; Richter, O. Ernährung der Algen. 1911.

³ Pascher, A. Ber. bot. Ges. 33: 427. 1915.

⁴ Harder, K. Zeitschr. f. Bot. 9: 145. 1917.

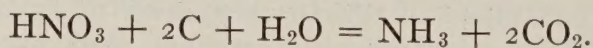
⁵ Maertens. Diss. Halle. 1914.

⁶ Charpentier. Compt. rend. 134: 672. 1902; cf also Klebs. Untersuch. d. bot. Inst. Tübingen. 2: 538. 1888; de Vries, H. Bot. Zeit. 46: 229. 1888; Richter, O. Ber. bot. Ges. 21: 493. 1903.

from amino acids, fatty acids, hydroxy acids and even from urea, hydantoin, creatin and similar substances.¹ The high nutritive value of fatty acids for green algae is of special interest.² The algae thereby take an intermediate position between seed-plants and microorganisms without chlorophyll. The fact is worth noting that in respect to artificial feeding, the *Cyanophyceae* are closer to the higher plants than the green algae; in darkness they feed only on carbohydrates and polyvalent alcohols. In this case the hexavalent alcohols are less useful to them than sugars.

Particular attention should be given to the fact that the assimilation of CO₂ in the light frequently continues undisturbedly when the algae are fed organic substances.³ It is not easy to estimate the reliability of the contradictory statements that algae have been artificially induced to adopt a purely saprophytic mode of living. In this connection unlike results have been obtained with different algae;⁴ especially in the *Cyanophyceae* is the chlorophyll preserved in darkness.⁵ Among others there is the following interesting experiment with *Scenedesmus*;⁶ for 8 years this alga has been grown on sugar with light excluded and not only does the chlorophyll remain unchanged but O₂ is at once liberated when the alga is exposed to light.

The nitrogen nutrition of the lower green plants as well as of the higher ones can take place at the expense of inorganic nitrogen compounds in cultures reared on organic substances. In the light the seed-plants assimilate nitrates better than ammonia but in darkness, according to reliable information, the opposite is true.⁷ In the dark, the algae can reduce nitric acid to ammonia⁸ and consequently they use nitrates just as the molds do (p. 187). In this case the oxygen split off from the nitric acid is liberated in the form of CO₂ because the carbon of the respiratory material is burned by this oxygen:



This "extra carbonic acid" is, according to Warburg, easily distinguished from the carbonic acid of normal respiration because respiration is 20,000 times less sensitive to the toxic effect of hydrogen cyanide. Thus by the proper use of the poison, the liberation of the extra CO₂ can be stopped without retarding normal respiration.

Some algae are said to assimilate ammonia as well as nitrates in the light,⁹ but it is uncertain whether photosynthesis continues in these cases. On the

¹ *loc. cit.*

² Treboux. Ber. bot. Ges. 23: 432. 1905.

³ Loew und Bokorny. Jour. f. prakt. Chem. 144: 272. 1887; Bokorny. Ber. bot. Ges. 6: 116. 1888; 9: 103. 1891; Idem. Biol. Centralbl. 17: 1. 1897.

⁴ Radais. Compt. rend. 130: 793. 1900; Matruchot et Molliard. Ibid. 131: 1248. 1900; Idem. Rev. gén. bot. 14: 113. 1902; Artari. Ber. bot. Ges. 20: 172. 1902.

⁵ Bouilhac. Compt. rend. 125: 880. 1897; 133: 55. 1900; cf. also Pringsheim, E. Cohns Beitr. Biol. d. Pflanzen. 12: 49. 1913.

⁶ Dangeard. Compt. rend. 172: 254. 1921.

⁷ Petrow, G. G. *loc. cit.*

⁸ Warburg, O. und E. Negelein. Biochem. Zeitschr. 110: 66. 1920; Muenscher, W. C. Bot. Gaz. 75: 249. 1923.

⁹ Benecke, W. Bot. Zeit. 56: 89. 189 Charpentier n. 8V Inst. Pasteur. 17: 321. 1903; Chick. Proc. Roy. Soc. London. 71: 458. 1903.

whole some algae, such as the gonidia of lichens,¹ occupy a middle position between seed-plants and microörganisms without chlorophyll in respect to nitrogen nutrition, because they prefer peptone to inorganic nitrogen compounds. The source of nitrogen for many algae can be various substances which cannot be used by seed-plants, such as urethane, amines, pyridine and alkaloids.²

In respect to the artificial feeding of moss leaves, it has been proved that starch is formed in them when fed with sugar but not when fed with acids.³ Analogous observations have been made with the protonema of various mosses.⁴ It has been shown that *Leptobryum piriforme* can develop on purely mineral solutions.

3. THE NUTRITION OF THE INSECTIVOROUS PLANTS

We know that the insectivorous or carnivorous plants contain chlorophyll. Thus they have photosynthesis like other seed-plants but in addition they have certain organs for catching animals and digesting meat. Oftentimes these organs are complicated and ingeniously formed; they are divided into

I. Pitchers and vases which contain digestive juices and play the rôle of animal traps (*Nepenthes*, *Sarracenia*, *Darlingtonia*; also *Utricularia*);

II. Glands with sticky secretions to which the insects adhere and undergo digestion (*Drosera*, *Drosophyllum*, *Pinguicula*);

III. Animals traps which are capable of rapid movements (*Dionaea*, *Aldrovandia*).

This is not the place to consider the arrangement and function of all these apparatus which have been well described by Ch. Darwin⁵ and other writers.⁶ The characteristics of the insectivorous plants are explained in all textbooks of general botany. Two questions will be considered here. In the first place, how necessary is it for the insectivorous plants to have a meat diet? and, secondly, what is the principal biochemical significance of the digestion of animals? The first question may be regarded as answered. Ch. Darwin showed that *Drosera* and *Pinguicula* are just as capable of photosynthesis as non-carnivorous plants.⁷ On the other hand, it has been found that *Drosera* and *Utricularia* grow much better with a meat diet.⁸ Taking all these facts together, one concludes that the insectivorous plants require the meat for its nitrogen and mineral salts rather than for the carbon. The fact is that the moor lands, on which the carnivorous plants of our habitats usually grow, are

¹ Beijerinck. Arch. néerland. de physiol. de l'homme et des anim. 24: 278. 1891; Idem. Centralbl. f. Bakt. 13: 368. 1893; Artari. Abh. d. Moskauer Naturforscherges. Vol. 6. 1899.

² Loew und Bokorny. Jour. f. prakt. Chem. 36: 272. 1887; Lutz. Ann. Sci. Nat. (7) 1: 75. 1899.

³ Marchal, El. et Em. Bull. Soc. Bot. Belg. 43: 115. 1906.

⁴ Goebel, K. v. Beih. bot. Centralbl. 21 (I): 325. 1907; Laage. Ibid. Vol. 76.

⁵ Darwin, Ch. Insectivorous Plants. 1875.

⁶ Goebel, K. v. Pflanzenphysiol. Schilderungen. 1891; Kerner. Pflanzenleben. I; detailed biological data on the *Nepenthaceae* are given by Stern, K. Flora. 109: 213. 1917.

⁷ Kostytschew, S. Ber. bot. Ges. 41: 277. 1923. The opposite results of Schmidt, G. Flora. 104: 335. 1912, and of Ruschmann, G. Diss. Jena. 1914 are not based on quantitative measurements. *Utricularia* likewise liberates much oxygen in the light.

⁸ Darwin, Fr. Jour. Linnean Soc. 17: 17. 1878; Rees, Kellermann und Raumer. Bot. Zeit. 36: 209. 1878; Büsgen, M. Bot. Zeit. 41: 569. 1883; Idem. Ber. bot. Ges. 6: LV. 1888.

very poor in nitrogenous foods. Peat bogs are usually very poor in mineral salts as well.

Since the pitchers of *Nepenthes* and the leaves of *Drosera* digest not only living insects but also small pieces of meat, fibrin and similiar substances, the older investigators assumed the presence of very active proteolytic enzymes in these plants. This hypothesis has not been confirmed. From the juices of both the *Nepenthes* pitchers and the *Drosera* leaves¹ there have been isolated active enzymes which apparently belong chiefly in the group of proteases seldom found in plants. These enzymes split protein only as far as peptones² (like the enzymes of the gastric juice) but not to crystalline products as is usually the case in plants. In *Pinguicula* the protein digestion goes in part as far as the formation of amino acids.³ The presence of free acid is necessary for the proteolytic enzymes of the pepsin type (Chap. VI). Accordingly the juices of *Nepenthes* and *Drosera* become acid when a protein is added,⁴ as the result of the secretion of free organic acids the nature of which is not yet known. The acids are secreted by glands of the leaves.

The proteolytic enzymes have not yet been demonstrated in other, less accurately studied carnivorous plants; even their existence has often been questioned. But since such doubts were at first expressed for *Nepenthes* and *Drosera*,⁵ but later removed, perhaps it is correct to assume that all carnivorous plants contain active proteolytic enzymes. There is already positive information in regard to *Sarracenia* but there is no reliable information yet about the rest⁶ except that negative results have been obtained recently for *Utricularia*.⁷

It is a remarkable fact that the insectivorous plants of our high moors are so different from our bog plants in that they have no mycorrhiza (p. 197). Perhaps this helps to explain the biological importance of their meat diet.

4. PROBLEMS OF PARASITISM AND SYMBIOSIS

Parasitism is usually defined as the method of nutrition by which the substances required by the parasitic organism are procured from the bodies of other organisms. Parasitism occurs in higher plants as well as in the lower ones. The chlorophyll-free fungi and bacteria often make no distinction between parasitic and saprophytic nutrition. Most of the parasitic and pathogenic (*i.e.*, causing a particular disease) fungi and bacteria are able to grow in pure cultures on solutions of various organic substances.

The lower organisms thus illustrate the close relationship between parasitism and saprophytic feeding on prepared organic substances. Of course, there is

¹ Vines, S. H. Ann. Bot. 11: 563. 1897; 12: 545. 1898; 15: 563. 1914; 23: 1. 1909; Clautriau. La digestion dans les urnes de *Nepenthes*. 1900.

² White, J. Proc. Roy. Soc. London (B) 83: 134. 1911; Abderhalden, E. und Teruuchi. Zeitschr. f. physiol. Chem. 49: 1. 1906.

³ Dernby, K. G. Biochem. Zeitschr. 80: 152. 1917.

⁴ Darwin, Ch. *loc. cit.*; Clautriau. *loc. cit.*

⁵ Tischutkin, N. Ber. bot. Ges. 7: 346. 1889; Dubois, R. Compt. rend. 111: 315. 1890; and others.

⁶ Gies, W. S. Jour. N. Y. Bot. Garden. 4: 38. 1893.

⁷ Kiesel, A. Ann. Inst. Pasteur. 38: 879. 1924. [See also Lloyd, F. E. Plant Physiol. 4: 87-102. 1929.]

no definite boundary between the two types of nutrition from the biochemical standpoint so that we, contrary to the older arbitrary conceptions, can find no *essential* distinction between parasitic and saprophytic nutrition.¹ The parasite feeds on prepared organic substances just like the saprophyte and must only produce, in addition to the "set of enzymes" and the other substances generally required for nutrition to be found in the saprophyte as well, some other accessory enzymes and poisons which, on the one hand, are used to dissolve the cell wall of the host plant,² and on the other hand, to poison the protoplasm of the host plant after which its nutritive substances become available to the parasite. In regard to the "social" side of the relations of the parasite to its host, this is nothing but an arbitrary application of human psychology to other organisms. At least this conception is not scientific and cannot be used as a working hypothesis.

The parasitism of seed-plants differs in some characteristic features from that of the lower plants. The less pronounced are the signs of autotrophic nutrition peculiar to seed-plants, the greater the morphological and histological degeneration in the development of all parts of the plant body with the exception of the organs of reproduction. Thus we see that under certain conditions it is actually possible for natural processes to replace the photosynthesis of seed-plants by nutrition with prepared organic compounds but not by experimental methods. The completely heterotrophic method of nutrition of parasites is explained by the lack of chlorophyll as a factor no longer required by the parasite and by the reduction of leaves as well as other organs and tissues which are involved in the normal nutrition through light and are only slightly developed in parasites. Parasitism among the lower organisms without chlorophyll involves no great change in their living conditions; for the green seed-plants it is much different and thus the question arises as to the primary cause of heterotrophic nutrition. Before we try to answer this question it will be necessary to consider carefully the various stages of parasitism among seed-plants.^p

The first stage of green parasites appears in the family *Scrophulariaceae* which includes the genera *Melampyrum*, *Alectorolophus*, *Bartschia*, *Tozzia*, *Euphrasia*, *Pedicularis* and *Odontites*. All these plants have normal aerial organs and contain an abundance of chlorophyll but their root systems are poorly developed. Only a few members of this group have root-hairs and then not many. In place of root-hairs we find haustoria with the help of which the roots of parasitic plants attach themselves to the roots of other plants and draw their foods from them.

The morphology and biology of green parasites have been studied in great detail.³ It has been found that there are gradual stages of parasitism among these plants. *Euphrasia* and especially *Odontites* have a certain number of

¹ On this point cf. A. Elenkin. Das Gesetz des beweglichen Gleichgewichts in Genossenschaften und Verbänden der Pflanzen. Mitt. Bot. Garten in Petersburg. Vol. 20. Pt. II. 1921.

² This name is given to the plant on which the parasite has established itself.

^p See also Skene, M. Biology of the Flowering Plants. P. 281-282. 1924 for other considerations of the origin of the parasitic habit.—Ed.

³ Koch. Jahrb. wiss. Bot. 20: 33. 1889; Heinricher. Ibid. 31: 77. 1898; 32: 389. 1898; 36: 665. 1901; 37: 264. 1902; 46: 273. 1909; 47: 539. 1910.

root-hairs and are therefore able to develop independently but to a limited extent. In *Alectorolophus* and *Bartschia* there are no root-hairs and these plants cannot ordinarily develop independently. In the choice of host plants these parasites are very unassuming and they often attack plants of the same kind, thus indicating an incomplete adaptation to parasitic nutrition. The species of *Melampyrum* have gone a step further because some of them prefer to live on different trees. *Tozzia* is practically a "holoparasite" which spends most of its life beneath the ground and is fed entirely by the host plant. From such observations it is clear that this group of plants takes the first step, so to speak, on the way to parasitism and does not yet possess the typical signs of true parasites. This makes the green parasites very interesting objects for physiological investigations. Accurate quantitative information concerning the nutrition of green parasites has been obtained recently for the first time.¹ It was found that, contrary to the prevailing idea,² the photosynthetic capacity of some species of *Melampyrum*, *Pedicularis*, *Alectorolophus*, *Odontites* and *Euphrasia* is not inferior to that of normal autotrophic plants. Likewise, there is a high rate of transpiration of green parasites under natural conditions on warm sunny days. The green parasites thus exhibit all the typical characteristics of plants nourished by photosynthesis and have large quantities of air and water vapor passing through their leaves. However, there proved to be a sharp distinction from normal green plants in respect to water absorbed from the soil by their roots. Even when the soil is completely saturated with water, the roots of the green parasites would be unable to meet the requirements for transpiration if they were to take the water directly from the soil and not from the host plant. The parasitism of these interesting plants is therefore very peculiar; they are forced to obtain from the host plant only the water which cannot be provided fast enough of themselves because of the lack of balance between root system and foliage.

Perhaps we should include in the same category *Viscum* and *Arceuthobium*, green parasites which root on the branches of trees. The evergreen leaves of *Viscum* assimilate CO₂ rapidly in the light (*loc. cit.*) but study of the haustoria shows that the parasite penetrates directly to the wood of the host plant where the water is passing upward.

On the basis of these observations and experimental results obtained with green parasites, it can be assumed that the first inducement to parasitism among seed-plants is lack of sufficient water. The unknown primary causes have limited the development of the root systems of green parasites so that they are insufficient for the requirements of sun plants. The green parasites of the *Scrophulariaceae* belong directly in the category of inhabitants of sunny, moist places.

The further advances in the parasitic habit of nutrition can be represented as follows. As the water is absorbed from the roots of the host plant, some

¹ Kostytschew, S. and E. Tswetkowa. Jour. russ. bot. Ges. 5: 21. 1920 (Russian); Kostytschew, S. Ber. bot. Ges. 40: 273. 1922; Idem. Beih. z. bot. Centralbl. 50 (I): 351. 1924.

² Bonnier, G. Compt. rend. 113: 1074. 1891; Idem. Bull. sci. du nord de la France et de la Belgique. 25: 77. 1893.

organic substances incidently reach the body of the parasite. From the examples of the green parasites just described, it can be seen that at first this condition plays no essential rôle but in the course of time the green parasite adapts itself to the partially relieved work of the chlorophyll apparatus and the chlorophyll is gradually lost, after which the leaves and then the vascular system gradually become atrophied. We find then a series of transformations of green parasites, starting with the reduction of the root system and ending with the extreme examples of complete parasitism. The pale green *Cuscuta Cephalanti* and *Cuscuta europea* liberate a small amount of oxygen in the light but they show a reduction of foliage and even of chlorophyll.¹ The substances synthesized in the light do not satisfy the vital requirements of these parasites so the haustoria of species of *Cuscuta* penetrate both bark and wood of their host plants. The representatives of the large genus *Orobanchë* are also unassuming parasites which attach themselves to certain plants by means of haustoria. Even the seed germination of species of *Orobanchë* can take place only near the roots of these host plants. Still *Orobanchë* liberates a small amount of oxygen in the light.² *Limodorum abortivum* also contains chlorophyll which has a weak action.³ All these facts indicate that the normal nutrition of seed-plants was at first gradually replaced by parasitic nutrition. Undoubtedly various *Balanophoraceae* and *Rafflesiaceae* have entirely lost the capacity for photosynthesis. Our *Monotropa* also lacks chlorophyll and does not liberate oxygen in the light,⁴ while the dark-brown *Neottia*, like the brown algae, contains a certain amount of chlorophyll.⁵ Neither *Monotropa* nor *Neottia* are attached to other plants by root haustoria; both grow on soils rich in humus and it is usually thought that they are parasites on the fungi of the soil. Some other types of seed-plants without chlorophyll also belong in this category of "mycotrophic" plants.⁶ The chemical side of the nutrition of phanerogamic parasites has been studied very little. It is only known that they form a certain amount of starch⁷ and that their protoplasm contains a considerable amount of lipoids.⁸ The osmotic pressure in the roots of parasites is often higher than in the host plant.⁹

Symbiosis, so-called, is usually sharply differentiated from parasitism. By this term we mean that two organisms, unlike in nature, live together to the mutual advantage of both "symbionts." Indeed it is often assumed that symbiosis differs from parasitism mainly in that there is no contest between symbionts which render mutual assistance to each other. According to this conception, symbiosis is intended to render mutual aid.

¹ Bonnier, G. *loc. cit.*

² Temme. Ber. bot. Ges. 1: 485. 1883; Molliard. Compt. rend. 147: 685. 1909; Ewart. Jour. Linnean Soc. 31: 446. 1895.

³ Josopait. Diss. Basel. 1900.

⁴ Griffon. Compt. rend. 127: 973. 1899.

⁵ Wiesner, J. Jahrb. wiss. Bot. 8: 575. 1872; Idem. Flora. Vol. 73. 1874.

⁶ Not all writers consider these plants to be mycotrophic parasites; some call them typical saprophytes.

⁷ Figdor. Ann. jard. bot. Bustenzorg. 14: 224. 1896; MacDougal. Contrib. New York Bot. Gard. 1899.

⁸ Biedermann, W. Flora. 113: 133. 1920.

⁹ Zellner, J. Monatschr. f. Chemie. 40: 293. 1919; Wosolsobe und Zellner. Sitzungsber. Akad. Wien, Math.-natur. Kl. IIb. Vol. 123. P. 1011.

If we refuse to accept the anthropomorphic idea of parasitism, we must also get rid of the conception of "mutualistic" symbiosis.¹ It originated at a time when there was no chemical physiology and our knowledge of plant life consisted entirely of the contents of so-called plant biology which makes use of neither exact measurements nor quantitative methods of investigation and indeed there is often no critical examination of its somewhat transcendental explanations. This subject has lived its life and its results must now be divided between plant physiology and ecology.

Above all it is erroneous to make use of the principle of external usefulness to the mutual relations of natural phenomena which we may observe;² hence it is quite wrong to add this supposed usefulness to the sociological character.³ The cases of so-called symbiosis which have been studied by exact methods have actually given a picture which fails to correspond to the "social" theory of symbiosis. The symbiosis of the *Leguminosae* with tubercle bacteria has been studied the best. We already know that the bacteria penetrate the bodies of the *Leguminosae* as typical parasites because they dissolve the wall of the root hair and thereby bring about a pathological curling of this organ. On its part, the seed-plant does a number of things to destroy the invading organism and often succeeds; then no tubercles are formed on the roots. The bacteria protect themselves from digestion by secreting a special membrane, just as parasitic bacteria often do. When the bacteria succeed in entering the root parenchyma, there is an effect of the protoplasm of the cells on the naked bacteria after it has shed its membrane. If the protoplasm of the seed-plant is strong enough to change the bacteria to bacteroids the normal tubercle develops but if the root is infected too late and the balance of power is on the side of the bacteria, the bacteroids do not form and the seed-plant suffers from the presence of the invader. Thus we have here a clear picture of the constant struggle for existence between the higher and lower plants, a typical picture of parasitism. If both sides gain as the result of this struggle (the *Leguminosae* are supplied with nitrogen and the bacteria return to the soil in greater numbers than when they entered the root tissue) this cannot be counted as of teleological nature.⁴ In connection with *Viscum*, it is not improbable that this evergreen parasite supplies its host with organic substances in the winter⁵ but that does not prevent us from considering the mistletoe as a true parasite.

According to Elenkin's results,⁶ there is exactly the same relationship between fungi and algae in the "lichen consortium" as that between legumes and the tubercle bacteria. The "mutualistic" theory of lichens as a peaceful partnership of mutually helpful organisms is challenged. It is more likely that

¹ Bernard, N. L'évolution des plantes. 1916: Elenkin, A. Mitt. d. bot. Garten in Petersburg. Vol. 20. Pt. 2. 1921.

² Kant, I. Kritik der Urteilkraft, Kant's Gesam. Schriften, Akad. Ausg. 5: 366. 1908. On the contrary it would be incorrect to assume that the principle of internal fitness should be banned entirely from biological science.

³ Elenkin, A. Ber. bot. Garten in Petersburg. 6: 1. 1906; Vol. 20. Pt. 2. 1921.

⁴ Kant, I. loc. cit.

⁵ Bonnier, G. loc. cit.

⁶ Elenkin, A. loc. cit.; also Mitt. bot. Garten in Petersburg. 2: 65. 1902; 4: 25. 1904.

the same result will be obtained in the future in all cases of symbiosis to the extent that they can be analyzed by quantitative experiments.

5. MYCORHIZA

Among the phenomena of symbiosis it is necessary to consider quite thoroughly the so-called mycorrhiza. Mycorrhiza are symbiotic unions of fungus hyphae and the roots of seed-plants, which occur with extraordinary frequency.

The mycorrhiza are endotrophic if the fungus penetrates the cells of the root and ectotrophic when the fungus forms a thick mat of its hyphae about the root. The endotrophic mycorrhiza of the *Orchidaceae* (Fig. 29) and the *Ericaceae* are especially interesting.¹ The fungal hyphae fill some cells of the root parenchyma of its host. Although the behavior of the fungus in many cases resembles that of a typical parasite and the seed-plant often digests its guest, the remarkable fact is that the presence of the mycorrhiza is necessary for the seed germination and tuber formation of the orchids.² The mycorrhiza are most widely distributed; they occur in at least half of our native seed-plants.

The ectotrophic mycorrhiza were discovered earlier than the endotrophic, on *Monotropa*.³ They are especially common on the various *Cupuliferae* and *Betulaceae* and likewise on some *Coniferae*. The development of the ectotrophic mycorrhiza causes a reduction of root-hairs. The latter appear to be replaced by fungus hyphae which grow between the particles of soil and humus (Fig. 30). The trees can also grow in the complete absence of the fungi and in these cases the mycorrhiza do not appear to be absolutely necessary.

Many mycorrhiza multiply only vegetatively and have not yet been accurately identified. They are known to include species of *Rhizoctonia* and fungi of the *Agaricaceae*, *Tuberaceae* and even *Boletus* type (*B. luteus*, *B. elegans*).⁴ Whether the mycorrhiza take any part in the nutrition of the plants

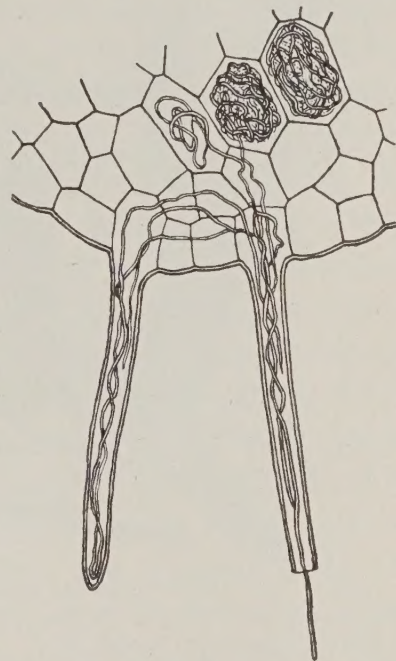


FIG. 29.—Endotrophic mycorrhiza of the orchid *Listera cordata*. The fungus enters the root through the root-hair and forms coils of hyphae in the cells of the cortex of the root. (After Chodat and Lendner.)

¹ Frank. Ber. bot. Ges. 5: 395. 1887; Burgef. Die Wurzelpilze der Orchideen. 1909; Kusano. Jour. Coll. Agr. Tokyo. Vol. 4. 1911; Arcularius. Centralbl. f. Bakt. (II) 74: 191. 1928.

² Bernard, N. Compt. rend. 138: 828. 1904; 140: 1272. 1905; Idem. Rev. gén. bot. 16: 405. 1904; [Ann. Sci. Nat. Bot. (9) 9: 1. 1909; 14: 235. 1911. For the application of Bernard's discoveries to the commercial growing of orchids from seeds, see Costantin and Magrou. Ann. Sci. Nat. Bot. (10) 4: 1. 1922. The germination of orchid seeds in cultures containing sugar but no fungus has been reported by Knudson (Bot. Gaz. 73: 1. 1922) who is also able to keep the plants growing on sterile media provided certain substances are supplied for the metabolism of the plants. For a summary of the question see: Knudson, L. Proc. Int. Cong. Plant Sciences, Ithaca, 1926. Vol. 2, pp. 1183-1189. R. V. LaGarde (Ann. Missouri Bot. Gard. 16: 499-514. 1929) reports complete confirmation of Knudson's methods which he has applied to certain seeds, but he also emphasizes the pH of the media as an important factor].

³ Kamensky, Th. Bot. Zeit. 39: 457. 1881.

⁴ Melin. Ber. Bot. Ges. 39: 94. 1921.

is uncertain. The hypothesis that mycorrhiza assimilate free nitrogen¹ has not been confirmed by a series of tests² except for members of the genus *Phoma*, which have been isolated from the roots of some of the *Vaccineae*.³ In this case it was found that these fungi are identical with the mycorrhiza of the plant.⁴ The *Vaccineae* and *Ericaceae* are actually able to get along with free nitrogen.⁵ Other workers think that seed-plants are able to absorb organic compounds of the soil by the aid of the mycorrhiza because the substances absorbed by the



FIG. 30.—Ectotrophic mycorrhiza. The fungus hyphae surround the root and displace the root-hairs. (After Frank and Tschirch.)

fungus pass into the protoplasm of the host plant. Stahl⁶ attributes to mycorrhiza a particular importance in the assimilation of minerals by seed-plants and bases this theory on the following observations. In soils rich in humus there is a lively struggle between the various kinds of microorganisms to obtain the mineral substances. Seed-plants which have no mycorrhiza cannot compete with the lower organisms and consequently do not grow as well.

It would be very desirable to undertake a thorough biochemical study of the question of the nutrition of plants with mycorrhiza. It is a very important problem in the nutrition of plants through the soil and it is fair to say that only after solving this problem shall we be able to obtain a correct idea of the nature of soil nutrition. This concerns the bog plants especially. The nitrogen nutrition of plants on the moor soils must be very peculiar. For the most part no nitrification takes place in moor soils and ammonia fertilizers prove to be entirely without effect. Yet almost all typical bog plants possess mycorrhiza. Various mosses also enter into symbiosis with fungi.⁷ Only by a careful biochemical study of the soil nutrition of marsh plants can we hope to clear up the dark secret of swamp life. The enormous theoretical and practical interest of the points involved require no explanation.⁸

¹ Frank. Landwirt. Jahrb. 19: 523. 1890; Schibata. Jahrb. wiss. Bot. 37: 643. 1902; and others.

² Möller. Ber. bot. Ges. 24: 230. 1906; Beijerinck. Bot. Centralbl. 104: 90. 1907.

³ Ternetz, Ch. Jahrb. wiss. Bot. 44: 353. 1907. Duggar, B. M. and A. R. Davis. Ann. Missouri Bot. Gard. 3: 413. 1916.

⁴ Rayner, M. C. Ann. Bot. 29: 97. 1915.

⁵ Idem. Bot. Gaz. 73: 226. 1922.

⁶ Stahl, E. Jahrb. wiss. Bot. 34: 539. 1900.

⁷ Janse. Ann. jard. bot. Buitenzorg. 14: 53. 1897; Nemec, B. Ber. bot. Ges. 17: 311. 1899; Golenkin, M. Flora. 90: 209. 1902.

⁸ For a more extended discussion of mycorrhiza see Rayner, M. C. Mycorrhiza. 1927, which is a reprint of a series of papers in New Phytologist.—Ed.

CHAPTER V

THE MINERAL NUTRITION OF PLANTS

1. THE GENERAL PHYSIOLOGICAL IMPORTANCE OF MINERAL SALTS

The physiological rôles of the organogens, *i.e.*, of carbon, oxygen, hydrogen and nitrogen, require no explanation. From these elements are formed carbohydrates and proteins which are the basis of living substance. The importance of compounds of phosphorus and sulphur has also been long recognized because these elements form a part of the protoplasmic proteins and lecithins but the rôles of the metals in relation to the organism were not understood for a long time. By the middle of the past century various opinions had been expressed, according to which the mineral salts are required only while the organism is developing; for the mature animals and plants they were said to be an unnecessary but unavoidable impurity in the normal foods. Such views were developed especially in animal physiology because the animal, unlike the plant, stops growing at a certain time; after that the food absorbed only serves to meet the current needs of life so the important factor is usually the number of calories furnished by its combustion. From this standpoint the mineral salts are unnecessary but their importance is much greater and many-sided. The exposition of their physiological rôles is one of the most brilliant applications of physical chemistry to physiology. In recent years the dynamic significance of mineral substances in the organism has come more and more to the foreground. The proteins and the rest of the substrate of vital phenomena are to be regarded as inert material "but the mineral substances represent machinery which regulates the working up of this material."¹ This view was expressed long ago but only in recent years has it received general recognition. To summarize all the information concerning the physiological rôles of the mineral substances, their importance can be stated as follows:

1. Mineral substances are active factors in altering the physical state of cell colloids, *i.e.*, they influence the inner structure of cells as well as the metabolism.
2. In connection with this action, they exert toxic and antitoxic effects on living tissues and organs.
3. In thus affecting the functions of tissues and organs, the mineral substances often act as hormones, *i.e.*, as regulators of physiological functions and the systematic development of organs. In animals the mineral substances are especially important in the sexual processes.
4. In many cases they function as catalysts of biochemical reactions.
5. They play an important rôle in varying turgor and protoplasmic permeability.

¹ Köppe, H. Die Bedeutung der Salze als Nahrungsmittel. 1896.

6. Oftentimes they are carriers of electrical and radioactive forces which are essential to the organism.

The physiological importance of the mineral substances is therefore surprisingly great. Only when this is correctly recognized is it possible to conduct an experimental investigation in the mysterious fields of physiology. The above mentioned ways in which mineral substances are important apply in principle to both plants and animals but in the preceding chapters we have noticed many differences in the nature of the nutrition of animals and plants. The most important facts in the realm of the physiological rôles of mineral substances were first demonstrated in animal physiology; later the validity of some of them for plants was proven. Accordingly it will be impossible to avoid all references to animals in the following discussions.

On account of the newness of the question of the importance of mineral substances, the specific physiological rôles of the single ash constituents has been investigated almost not at all. It is known that the same mineral substances are not essential to animals and plants alike and even among the plants there are certain variations in this respect. The older physiologists sought to ascribe to each ash constituent its biological and often specific rôle. All these explanations were arbitrary and entirely lacking in experimental support. The only correct way to approach the matter is to start with the fundamentals of the physiological rôles of mineral substances and to try to trace all special cases back to these fundamentals.

Liebig¹ was the first to emphasize the outstanding importance of mineral substances for both plants and animals. Boussingault² and Fürst zu Salm-Horstmar³ performed the critical experiments with plants. However, de Saussure⁴ had already developed the idea of the plants' need for mineral salts. In animal physiology the current views center about the classical experiments of Forster.⁵

2. ANTAGONISM OF THE EFFECTS OF IONS ON COLLOIDS AND ON THE PHYSIOLOGICAL STATE OF ORGANS AND THE ENTIRE ORGANISM

In the first chapter it was explained that the influence of electrolytes on colloids is to precipitate them from their solutions in the form of either solid particles or jellies (gels). It was also emphasized that in the case before us we are dealing with reactions of ions because the precipitation of hydrophobe colloids by the dissociation of the precipitating salt depends on the rate of migration of ions and the valence of the metal concerned, in that the monovalent metals have a much weaker effect than divalent or trivalent metals.⁶ It is also important that positively charged colloids are precipitated by anions and the negatively charged ones by cations. There is a reversed effect of ions after

¹ Liebig, J. v. *Die organische Chemie in ihrer Anwendung auf Agrikultur und Physiologie.* 1840.

² Boussingault, J. B. *Agronomie, chim. agr. et physiol.* 1: 1. 1860.

³ Fürst zu Salm-Horstmar. *Versuche und Resultate über die Nahrung der Pflanzen.* 1856.

⁴ Saussure, Th. de. *Recherches chimiques sur la végétation*, p. 240 and 321. 1804.

⁵ Forster, J. *Zeitschr. f. Biol.* 9: 297 and 369. 1873.

⁶ Schulze. *Jour. f. prakt. Chem.* 25: 431. 1882; 27: 320. 1884; Freundlich. *Zeitschr. f. physikal. Chem.* 44: 135. 1903; 73: 385. 1910; Mines. *Jour. of Physiol.* 42: 309. 1911.

the colloid loses its electrical charge.¹ Because there is clear experimental evidence of the importance of ionic effects in the precipitation of colloids, it is important to emphasize the so-called antagonism between monovalent and divalent cations in the precipitation of colloids. The first column of the following table² contains the amounts of SrCl_2 required to precipitate a certain quantity of arsenious sulphide, while the second column gives the amounts of SrCl_2 required to bring about the same effect *in the presence of different amounts of KCl*:

SrCl_2	4.20	SrCl_2	4.90 + KCl 0.30
SrCl_2	3.60	SrCl_2	5.55 + KCl 1.20
SrCl_2	2.60	SrCl_2	5.65 + KCl 2.70

The proteins belong in the category of hydrophile colloids but after being discharged they have properties similar to those of hydrophobe colloids (p. 14) and such a process undoubtedly takes place frequently in the living cell. In fact an antagonism between salts in precipitating and dissolving protein has been found³ but the so-called antagonism is clearly shown in the case of alterations in the solubility of lecithin.⁴ Here there is antagonism between sodium and potassium as well as between monovalent and divalent ions.

If the surface tension γ of water is 100, γ for the solution of lecithin used equals 76. The addition of salts of Na, K and Ca increase the value of γ for the lecithin solution, *e.g.*

$$1\text{M NaCl}, \gamma = 90.3; \frac{1}{32}\text{M KCl}, \gamma = 86; \frac{1}{64}\text{M CaCl}_2, \gamma = 86.3.$$

Combinations of these salts do not give additive effects and certain proportions give such an equilibrium that there is no change in the surface tension of the lecithin solution:

$$1\text{NaCl} + \frac{1}{50}\text{KCl} + \frac{1}{100}\text{CaCl}_2; \gamma = 76 \text{ for any concentration of the solution.}$$

$$1\text{NaCl} + \frac{1}{20}\text{KCl}; \gamma = 77-79 \text{ for any concentration of the solution.}$$

The antagonism of ionic effect on colloids is attended by a change in surface tension and is therefore an adsorption phenomenon. This is shown clearly by experiments on the adsorption of ions by cellulose and charcoal.⁵

It is very interesting and important that this same antagonism between monovalent and divalent cations affects their toxic action on tissues and organs. Connections between facts which at first sight seem to be quite unlike often provide interesting and fruitful working hypotheses. This is true in respect to antagonism between ions.

S. Ringer⁶ was the first to call attention to the rhythmic contractions of muscles in a solution of pure sodium chloride. In the blood we find the same content of sodium chloride but there it produces no similar action. The abnormal condition is done away with by adding small amounts of the calcium and potassium salts that are also contained in the blood. J. Loeb⁷ made

¹ Hardy. Jour. of Physiol. 33: 251. 1905; Idem. Zeitschr. f. physikal. Chem. 33: 385. 1900.

² Linder and Picton. Jour. Chem. Soc. (London) 67: 63. 1895.

³ Pauli. Hofmeisters Beitr. 5: 27. 1903; 6: 233. 1903.

⁴ Neuschlosz, S. M. Pflügers Arch. f. ges. Physiol. 181: 17. 1920.

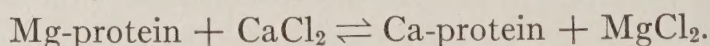
⁵ Rona, P. und L. Michaelis. Biochem. Zeitschr. 94: 240. 1919; 103: 19. 1920.

⁶ Ringer, S. Jour. of Physiol. 4: 29 and 222. 1883; 7: 291. 1886; 18: 425. 1895; and others.

⁷ Loeb, J. Ibid. 6: 411. 1902; and many others.

analogous observations with sea-urchins and jelly-fishes. Sea-urchin eggs do not develop in a solution of sodium chloride of the same concentration as sea water but this toxic effect of sodium chloride is decreased by the simultaneous action of any calcium salt or a salt of any divalent or trivalent metal. Loeb¹ attempted to connect this phenomenon with the foregoing effects of ions on colloids, because a series of control experiments showed that the effect of various salts on physiological processes is also due to the effects of single ions. At this point the facts became somewhat complicated in that some ions have specific effects and in addition there is antagonism between ions with the same valence. In spite of all this, the basic principles are essentially the same as in the precipitation of colloids. Reactions of ions are confined chiefly to two kinds of facts. In the first place, it has been established that the pharmacological effect of various inorganic poisons, such as salts of mercury, depends entirely on their dissociation and thus represents a reaction of ions,² and the toxic effect of salts is to be included in the same category of biochemical processes as the effect of protoplasmic poisons.³ Secondly, it is particularly the cations of salts which affect living cells with their negatively charged colloids of proteins and lipoids; the anions of these salts exert almost no effect on the antagonism of these cations. This agrees with the important principle of colloidal chemistry according to which the colloids and the ions which precipitate them must have electrical charges with opposite signs.⁴

To explain the specific secondary effects of separate ions, Loeb assumes that in the case of a toxic effect of ions on the proteins of protoplasm, there is a displacement of one ion by another from its union with the protein according to the law of mass action. For example, the following reaction takes place in the antagonism of calcium and magnesium, according to Loeb:⁵



The direction of this reaction is said to depend on the amount of Ca and Mg at any given time and the corresponding physiological effect is produced by the direction of the reaction. Loeb, who is joined by other workers,⁶ thus assumes, aside from the physical effect, purely chemical reactions which come close to explaining some specific effects (see below). Some chemical experiments⁷ support this theory but other investigators hold to the purely physical theory of neutralization of the electrical charge of colloids,⁸ or the displacement of one ion by another from the surfaces in heterogeneous systems.⁹

¹ *Idem.* Pflügers Arch. f. ges. Physiol. **71**: 457. 1898; **88**: 68. 1901; **91**: 248. 1902; *Idem.* Jour. of Physiol. **11**: 455. 1904; and many others.

² *Paul und Kronig.* Zeitschr. f. physikal. Chem. **12**: 414. 1896; *Idem.* Zeitschr. f. Hyg. und Infektionskrank. **25**: 1. 1897; *Grützner.* Pflügers Arch. f. ges. Physiol. **58**: 69. 1894; *Maillard.* Jour. de physiol. et de path. **1**: 651 and 673. 1899; and others.

³ There is even antagonism between metallic ions and alkaloids, the unquestioned poisons of protoplasm.

⁴ *Hardy.* Jour. of Physiol. **24**: 301. 1899; **33**: 251. 1905; *Idem.* Zeitschr. f. physikal. Chem. **33**: 385. 1900.

⁵ *Loeb, J.* Jour. Biol. Chem. **1**: 427. 1906.

⁶ *Robertson, T. B.* *Ibid.* **1**: 279 and 507. 1906; **2**: 317. 1907; *Idem.* Pflügers Arch. f. ges. Physiol. **110**: 610. 1905; *Richards, T. W.* Jour. Phys. Chem. **4**: 207. 1900.

⁷ *Osborne, W. A.* Jour. of Physiol. **33**: 10. 1905; **34**: 84. 1906.

⁸ *Mathews, A. P.* Amer. Jour. Physiol. **10**: 290. 1904; **11**: 237 and 455. 1904; **14**: 203. 1905; **18**: 58. 1907.

⁹ *Neuschlosz.* Pflügers Arch. f. ges. Physiol. **181**: 17. 1920.

In any case the analogies between the effect of ions on colloids on one hand and on living organs and whole organisms on the other, can be carried so far that they command attention in explaining the toxic and regulatory effect of mineral substances on various physiological processes. Physiological antagonism between ions can be explained by a large number of facts. The pertinent material has been largely furnished by American investigators. The specific effect of ions on muscular contraction is illustrated by many facts which explain both the effect of the inorganic constituents of the blood and the analogous effect of the compounds of sea water.¹ The results also apply to nervous tissue.² The antagonism between calcium and sodium which was pointed out by Ringer (see above) apparently has a general significance. Moreover antagonism between other ions of like or unlike valence has been shown by splendid experiments. Then was discovered the interesting antagonism between ions and alkaloids³ and (for plants) that between metals and H ions.⁴ On the basis of various analogous observations there grew up the concept of the "balanced solution."⁵ This refers to a solution of different salts in which the toxic effect of each metal is neutralized by the antagonistic effect of other ions, so that the solution as a whole exerts no toxic effect on living tissue. The first of these solutions was prepared by Ringer. It had the following *molecular* composition. For each 100 mols of NaCl take 1.7-2.4 mols of KCl and 1.1-2.4 mols of CaCl₂ and dilute the mixture as needed. Still more completely balanced is the so-called van't Hoff solution of the following composition: to every 1000 parts of 1.N NaCl solution add

1.N MgCl ₂	78 parts
1.N MgSO ₄	38 parts
1.N KCl.....	22 parts
1.N CaCl ₂	10-20 parts

The concentration of this solution can also be varied. In the search for balanced solutions, the remarkable fact was discovered that the proportions of cations in these solutions is the same as in sea water and in the blood of animals. Both sea water and blood are excellent balanced solutions of mineral salts. As Loeb puts it "we carry in our lymph and in the blood a small portion of the ocean in which our ancestors lived."

The antagonism of ionic effects on the separate physiological functions of plants and on the duration of life in mineral solutions is based on a large number

¹ Loeb, J. Anniv. vol. for Prof. Fick, p. 101. 1899; *Idem.* Amer. Jour. Physiol. 3: 327 and 383. 1900; *Idem.* Pflügers Arch. f. ges. Physiol. 91: 248. 1902; *Idem.* Jour. Biol. Chem. 1: 427. 1906; and others.

² Loeb, J. *loc. cit.*; Parker, G. H. and C. R. Metcalf. Amer. Jour. Physiol. 17: 55. 1906; Robertson, T. B. Trans. Roy. Soc. South Australia. 29: 1. 1905.

³ Pickering. Jour. of Physiol. 14: 383. 1893; 20: 165. 1896; Buchanan. *Ibid.* 25: 137. 1899; Zoethout. Amer. Jour. Physiol. 2: 220. 1899; and many others [including Robertson, T. B. Jour Biol Chem. 1: 507. 1906 and Osterhout, W. J. V. J. Gen. Physiol. 1: 515. 1919].

⁴ Domontowitsch. Tagebuch d. I. Kongr. d. russ. Botaniker, p. 37. 1921 (Russian).

⁵ For a statement of the origin of the concept of balanced solutions, see Loeb, J. Jour. Biol. Chem. 34: 503. 1918.—*Ed.*

of facts. The tables explained below are taken from papers by Osterhout.¹ The first table gives the duration of life of the marine plant *Ruppia maritima* in different solutions. The experiment was stopped at the end of 150 days and the + sign indicates that the plants were living normally at the close of the experiment.

Sea water.....	158+
$\frac{3}{8}$ N NaCl.....	23
$\frac{3}{8}$ N KCl.....	56
$\frac{3}{8}$ N CaCl ₂	58
$\frac{3}{8}$ N MgCl ₂	19
NaCl + KCl + CaCl ₂	88
van't Hoff's solution.....	150+

The following table gives the total growth of the roots of wheat seedlings in millimeters after 40 days in different solutions.

0.12N NaCl.....	59
0.12N KCl.....	68
0.12N MgCl ₂	7
0.12N CaCl ₂	70
1000NaCl + 10CaCl ₂ + 22KCl.....	324
van't Hoff's solution.....	360

The following table illustrates the antagonistic effect of potassium and magnesium on the duration of life of the liverwort *Lunularia*. The experiment was closed after 120 days and the + sign indicates that the plant material was normal at the close of the experiment.

Distilled water.....	120+
0.093N KCl.....	12
0.093N MgCl ₂	4
100KCl + 100MgCl ₂	120+
100KCl + 50MgCl ₂	120+
50KCl + 100MgCl ₂	120+

This work supports the idea of toxic and antagonistic effects of ions in the case of different materials and physiological functions.² The respiration of microörganisms also follows the general law of toxic effects³ and the same applies to tropisms.⁴ We are therefore entitled to attribute a general significance to the phenomena here described.

According to Loeb and Osterhout, the explanation of the physical antagonism of salts lies in the joint effect of the salts on the protoplasmic surface which is thereby made impermeable while the single salts easily penetrate the protoplasm and there bring about a toxic effect. Some writers⁵ have recently proposed

¹ Osterhout, W. J. V. Univ. of Calif. Publ. (Bot.) 2: 231 and 235. 1906; 3: 331. 1908; *Idem*. Bot. Gaz. 42: 127. 1906; 44: 259. 1907; 45: 117. 1908; 47: 48. 1909; *Idem*. Jahrb. wiss. Bot. 46: 121. 1908; 54: 645. 1914; *cf.* also Micheels. Compt. rend. 143: 1181. 1906; *Idem*. Arch. Inter. de Physiol. 4: 410. 1907; Benecke. Ber. bot. Ges. 25: 322. 1907.

² Kaho, H. Biochem. Zeitschr. 167: 25 and 182. 1926.

³ Brooks, M. M. J. Gen. Physiol. 3: 337 and 527. 1921; Gustafson, F. G. *Ibid.* 2: 17. 1919.

⁴ Cholodny, N. The influence of metallic ions on the irritant phenomena of plants. 1918 (Russian).

⁵ Neuschlosz, S. M. *loc. cit.*

another explanation. The antagonism of ions is due to an ionic effect within the protoplasm itself. The ions are said to displace each other from the adsorbed condition. The displaced ion is not replaced at the surfaces by the displacing ion and the colloid is left unchanged.^s

3. THE IMPORTANCE OF MINERAL SUBSTANCES AS HORMONES, CATALYSTS AND CARRIERS OF ELECTRICAL AND RADIOACTIVE ENERGY

All of the facts described above furnish data for estimating the rôle and the importance of mineral substances in the *normal* physiological processes of organisms. The effect of ions on colloids has been proved in the field of physical chemistry. When we consider that the structure of protoplasm depends on the physical condition of the cell colloids, because protoplasm is either a system of gels in a foam or network structure (p. 15) or a combination of liquid phases with unequal inner friction, etc., it is quite possible that changes in the medium are the causes of changes in the various biochemical processes. From the foregoing explanations, various toxic effects of unbalanced solutions of mineral salts may conceivably be a result of changes in solubility of the various protoplasmic colloids.

Antagonism of ions may regulate the course of various physiological processes as well as the condition of the cell colloids. Of course, the nature of the development and structural formation of the organism depends on the working of various biochemical processes. Thus we could assume that the regulatory effect of ions has the function of hormones under normal conditions. Hormones are substances which give the signal, so to speak, for the coördination of definite physiological processes and to provide for the systematic development of organs. Thus in animals, the liver and pancreas become active during digestion in the stomach and they secrete their specific juices in preparation for the digestion in the intestine. The signal to secrete these juices is not given through the nervous system but by means of movements of hormones. In the same way, when the apex of the coleoptile is stimulated by light from one side, a hormone passes to the place where phototropic curvature must occur. There the effect of the hormone is that of a stimulus. The planning (*Anlegen*) and development of whole organs is also accomplished through the agency of specific hormones.

In the literature there are numerous statements about the *specific* effects of some ions on various vital processes. Among others, the observations of Loeb on artificial parthenogenesis¹ are especially striking. Unfertilized sea-urchin eggs develop to normal larvae through the effect of calcium ions. On

^s An electrostatic theory of antagonism, based on permeability studies, has been proposed by Raber (Bot. Gaz. 75: 298. 1923; 81: 348. 1926). It is assumed that the protoplasm is negatively charged while the cell sap has a positive charge. Salts are considered as "positive" or "negative" according as the concentration of the charges on the ions is greatest on the cations or the anions. The conditions which result from the exposure of cells to solutions of individual salts or of mixed salts are said to be controlled by the electrical relationships between the various members involved. Both toxicity and antagonism, and even the composition of artificial nutrient solutions, are said to be accounted for by this theory. For a purely chemical explanation of antagonism between ions see Simms, H. S. J. Gen. Physiol. 12: 214, 259, 511 and 783. 1928-29.—Ed.

¹ Loeb, J. Untersuchungen über künst. Parthenogenese. 1906; [see also Studies in General Physiology. Part 2, p. 576 ff. 1905].

the contrary, the sodium ions retard such a parthenogenetic development but this effect can be lowered to any degree by calcium ions or even entirely annulled. No less remarkable are the observations of the same worker¹ concerning the development of dwarf and giant larvae and of paired embryos of *Arbacia*, the sea-urchin. All these deformities are experimentally produced with ease by using an unbalanced solution of mineral substances. It is astonishing that an excess of $MgCl_2$ in the sea water produces one-eyed (cyclops) embryos of the fish *Fundulus heteroclitus*.² It has been proved that the causes for the fusion of the two eye "*anlagen*" are the unbalanced magnesium ions. The development of organisms has thus been directed in different, predictable ways under the influence of ions. Loeb emphasizes expressly that a normal development of the sea-urchin occurs only when the proportions of sodium, potassium and calcium ions correspond to the normal conditions in sea water. The influence of sodium ions on the rhythmical contractions of skeletal muscles has been mentioned before. The calcium ions serve to regulate and check this peculiar effect of sodium on the nerves and muscles. In Loeb's opinion,³ all our skeletal muscles are prevented from contracting rhythmically as the heart muscles do, only by the presence of calcium in the blood.

Such cases are numerous in the field of animal physiology where there have been many studies of the specific effects of ions on various vital processes. However, analogous phenomena are also found in plant physiology. For example, there is the so-called "oligodynamic" or toxic action of different metals.⁴ A good illustration of the peculiar effect of ions on an important life process is the prevention of spore formation in the fungus *Aspergillus niger* by the presence of the slightest amount of zinc salts. This effect can be produced by no other metals and can be observed only when fed on sugar.⁵ The influence of mineral ions on changes in permeability and on the osmotic properties of living plant cells will be considered in Chap. IX which is devoted to this question.[†] It can hardly be doubted that all such effects are produced by various changes in the physical condition of the protoplasmic colloids. If this is actually so, it would seem that it is possible to regulate permeability and of course this plays a prominent rôle in the process of plant nutrition.

The catalytic effect of different metals has been observed many times. Bredig⁶ described it in its simplest form. It has long been known that salts of iron and manganese as oxidizing agents are similar to the oxidizing enzymes or peroxidases. Recently the view has been expressed that the active factors in the case of many oxidation processes in the plant cell are not true enzymes

¹ Loeb, J. Pflügers Arch. f. ges. Physiol. 55: 525. 1894; Idem. Amer. Jour. Physiol. 4: 423. 1901; Idem. Arch. f. Entwicklungsmechanik d. Organismen. 27: 120. 1909.

² Stockard, C. Jour. of Exp. Zool. 6: 285. 1909.

³ Loeb, J. Univ. of Chicago Decennial Publ. 10: 3. 1902.

⁴ Nägeli, C. Denkschr. f. Schweizer Naturforscher-Ges. 33: 1. 1893; Loew, O. Landwirt. Jahrb. 20: 235. 1891. Loeb explains all these facts on the basis of this theory.

⁵ Butkewitsch. Biochem. Zeitschr. 129: 445. 1922.

[†] This will be the first chapter in the second volume of the author's original work.—Ed.

⁶ Bredig. Anorganische Fermente. 1901.

but compounds of iron, the so-called haematoids.¹ The reducing enzymes or reductases are likewise relatively less specific² and their effect is analogous to that of some inorganic compounds. When sugar is heated with a small quantity of ferrous salts and soda, the result is a complex compound of iron which has definite reducing properties.³ It changes nitrobenzene to aniline and nitrous acid to ammonia. Baudisch⁴ reports that inorganic ferrous compounds as a whole have reducing properties.

Unfortunately it is only recently that the catalytic effect of metals in the organism⁵ has been taken into consideration although there have been various analogies in the field of inorganic chemistry.⁶ Recently there have come to be known other cases⁷ of complicated effects of ions which illustrate the physiological importance of mineral substances as stimulators.⁸ The latest work of A. J. Smirnow⁹ shows that various cations retard the action of catalase and divalent cations especially are powerful stimulators to the action of peroxidase, even in minimum concentrations. Zn occupies a special position in that it is extraordinarily effective in checking the action of peroxidase. Zn and Cd have the same influence on invertase and reductase.¹⁰ From the fact that ions can be carriers of electrical energy it is evident that the plasma membrane is sometimes unequally permeable to anions and cations. Some writers believe that such phenomena may be considered as sources of electromotive forces of organisms.¹¹

The unequal adsorption of different ions can also produce electrical forces as indicated on p. 6. In addition, radioactive processes have been described recently, which seem to be related to the antagonism of ions.¹² It is uncertain as to how far this statement will be confirmed. In any case it concerns the plants.¹³ It has been found that potassium radiation stimulates seed germination¹⁴ and that uranium salts promote the assimilation of free nitrogen by *Azotobacter*.¹⁵

¹ Gola, G. Atti d. Reale accad. dei Lincei rendiconto. 28: 146. 1919. [This idea is closely related to that expressed by Warburg, O. Science. 61: 575-582. 1925 in which some unnamed compound of iron is suggested as the carrier of oxygen for such "enzymes." Later work reported by Warburg and Negelein. Biochem. Zeitschr. 202: 202-228. 1928 confirmed this theory. The latest results were obtained from spectrum analyses.]

² Bach, A. Compt. rend. 164: 248. 1917.

³ Baudisch, O. Ber. chem. Ges. 50: 652. 1917.

⁴ Baudisch, O. und P. Mayer. Biochem. Zeitschr. 107: 1. 1920.

⁵ Baudisch, O. Ibid. 106: 134. 1920.

⁶ Wurster. Ber. chem. Ges. 22: 1910. 1889.

⁷ Courtonne, H. Compt. rend. 171: 1168. 1920; and others.

⁸ Kuhn, O. Jahrb. wiss. Bot. 57: 1. 1916; and others.

⁹ Smirnow, A. J. und S. P. Alissowa. Biochem. Zeitschr. 149: 63. 1924; Smirnow, A. J. Ibid. 155: 1. 1925.

¹⁰ Kostytschew, S. und S. Zubkova. Zeitschr. f. physiol. Chem. 111: 132. 1920; Kostytschew, S. und G. Medwedew. Zeitschr. f. physiol. Chem. 164: 77. 1927.

¹¹ Ostwald, W. Zeitschr. f. physikal. Chem. 6: 1. 1890.

¹² Zwaardemaker and Fenestra. Proc. Roy. Acad. Amsterdam. 19: 99, 341 and 633. 1916; Zwaardemaker and Lel. Arch. néerland. de physiol. de l'homme et des animaux. 1: 745. 1917; Zwaardemaker. Pflügers Arch. f. ges. Physiol. 169: 122. 1917; 173: 28. 1919.

¹³ Blackman, V. H. Ann. Bot. 34: 299. 1920.

¹⁴ Stoklasa, J. Biochem. Zeitschr. 108: 109, 140 and 173. 1920.

¹⁵ Kayser, M. Compt. rend. 172: 1133. 1921.

4. THE ARTIFICIAL FEEDING OF PLANTS WITH MINERAL SUBSTANCES

Since the green plants make their organic compounds out of carbon dioxide, water and inorganic salts, it has long been thought that seed-plants might be grown in the complete absence of organic substances. The success of such experimentation would help to explain the geochemical rôle of green plants and to disprove the humus theory of plant nutrition. For these reasons Liebig tried to grow seed-plants on purely mineral media but he encountered serious technical obstacles and the problem was not solved until later.¹ The method of artificial cultures in mineral nutrients is now so completely worked out that crops are obtained which are no smaller than those which are grown in agricultural operations on the most fruitful, natural soils. Artificial cultures have an inestimable practical and theoretical importance because there is no other way to analyze the normal conditions of plant nutrition. Because one can thus replace the complicated soil, with its large amount of substances of unknown composition, by a simple solution of mineral salts, it is possible to determine the needs of the plant for various substances by means of exact, quantitative analyses, and thus to find which of the ash constituents^u are essential components of the living substance. We can follow these elements from the time of seed germination until growth stops under any external conditions, and become intimate with the most important question of nutrition through the roots.

For reasons mentioned above, the so-called growth experiments, *i.e.*, artificial cultures of seed-plants carried all summer, are a powerful weapon of the present-day, scientific agricultural chemistry and are being used at every scientifically managed agricultural experiment station. The introduction of different methods of growing pure cultures of seed-plants is to be regarded as a means of furthering growth experiments. As it will be shown in the next chapter, some physiological problems can be solved only by using pure cultures.

5. METHODS FOR ARTIFICIAL CULTURES OF SEED-PLANTS

The seeds are first germinated in moist sawdust or on damp filter paper. When the roots are 2–3 cm. long, the seeds are fastened to the cover or stopper of a large culture vessel in such a way that the root enters the vessel and the shoot remains in the air. In order to avoid marked changes in reaction as the result of unequal absorption of single ions by the roots and to prevent loss of equilibrium between ions, the solution is very much diluted^v and used in large

¹ Wiegmann und Polstorff. Über die anorganischen Bestandteile der Pflanzen. 1842: Fürst zu Salm-Horstmar. Jour. Pract. Chem. 46: 193. 1849; Boussingault. Agronomie, chim. agr. et physiol. 1: 1. 1860; Sachs, J. Landwirt. Versuchs-Stationen. 2: 22 and 224. 1860; Knop. Ibid. 2: 35. 1860; 3: 295. 1861; 30: 292. 1884; Hellriegel und Wilfarth. Untersuchungen über Stickstoffnahrung der Gramineen und Leguminosen. 1888; Wortmann. Bot. Zeit. 50: 640. 1892; and others.

^u Qualitative analyses of the ash content of different plants have shown that the following 31 elements have appeared in the ash: aluminium, arsenic, barium, boron, bromine, calcium, chlorine, cobalt, copper, fluorine, iodine, iron, lead, lithium, magnesium, manganese, mercury, nickel, phosphorus, potassium, rubidium, selenium, silicon, silver, sodium, strontium, thallium, tin, titanium and zinc.—Ed.

^v For a thorough study of the effect of salt proportions and concentrations on the plant growth see Tottingham, W. E. Physiol. Res. 1: 133–245. 1914. J. W. Shive has also made a study of the ratios between the ions in these nutrient solutions (Physiol. Res. 1: 327–397. 1915). In order to avoid a change in the reaction of the solution about the roots, it is necessary to renew the solutions at certain intervals. Another way to keep all the factors of the solution constant is to allow the solution to flow slowly through the culture vessel. On this point see Trelease, Sam F. and B. E. Livingston. Science. 55: 483–486. 1922.—Ed.

quantities (the vessel should hold 10–25 liters; the larger it is, the better it will be for the culture). Experience has shown that even a concentration of 0.5% of mineral salts is toxic to the root system.¹ The vessel is either directly filled with the salt solution (water culture) or filled with ignited quartz sand previously washed in strong mineral acids (sand cultures). This treatment of the sand removes all soluble compounds. The sand is then watered with the salt



FIG. 31.—Greenhouse for growth experiments. Explanation in text. (After Timiriareff.)

solution. In order that there be no lack of oxygen for the respiration of the roots, fresh air is bubbled through the containers from time to time. The walls of the vessel must be opaque in order to prevent the growth of algae. The best containers are those of porcelain or zinc, made especially for artificial cultures.

Growth experiments must be so handled that they receive plenty of light. For this reason a greenhouse for growing plants is an essential part of the equipment of every well organized experimental station and laboratory of plant physiology. The greenhouse must be placed beside an unshaded site to which the culture vessels can be taken from the greenhouse by little wagons on rails (Fig. 31). The greenhouse itself must consist of clear window panes and all the frames of the roof as well as the side walls must be made to open and close. Linen screens are used to protect the plant from too strong sunlight. Because the cultures may be kept

¹ Nobbe. Landwirt. Versuchs-Stationen. 9: 478. 1867.

either in or out of the house according to the conditions outside, all kinds of plants including trees can be grown with certainty.^w

The principle of pure cultures consists in freeing the seeds of microorganisms and placing the cultures in sterilized vessels. The first experiments of this kind were undertaken by the French agricultural chemist Mazé.¹ Many improved experimental devices were later introduced.² It is impossible to describe here all the precautions for pure cultures. In general there are always two principal pieces of apparatus—one to sterilize the seeds and the other the culture vessel itself. Figure 33 shows an example taken from the work of G. G. Petrow. In tube A the seeds are sterilized with a 0.5% solution of sublimate [of mercury] or a 1% solution of bromine. They are then rinsed several times with sterile water. The tube of seeds is then inserted in the opening in the lid of the culture jar. The vessel containing the nutrient solution must first be sterilized. Then the two vessels are joined in accordance with the rules of bacteriological technique. The seeds fall into the culture vessel when the pinchcock *q* on the rubber tube is opened. They are spread on the sieve by the aid of the regulator so that the roots pass through the netting into the nutrient solution. The other tubes are closed with wadding to prevent an infection of the culture and are used later to pass air through the vessel and the nutrient solution.

In spite of all these precautions, pure cultures of the seed-plants are often not obtained because the seeds can be disinfected only superficially without destroying their capacity to germinate but the bacteria are often located under the seed coats where they existed on the parent plant. An infected culture can be recognized by its appearance after a few days and withdrawn from the series of experiments. That it is possible to run pure cultures of seed-plants for a long time is shown by the fact that the growth experiments succeed in the dark in the presence of organic nutrients.³ Under these conditions there should be an infection if only a couple of bacteria were present in the solution.

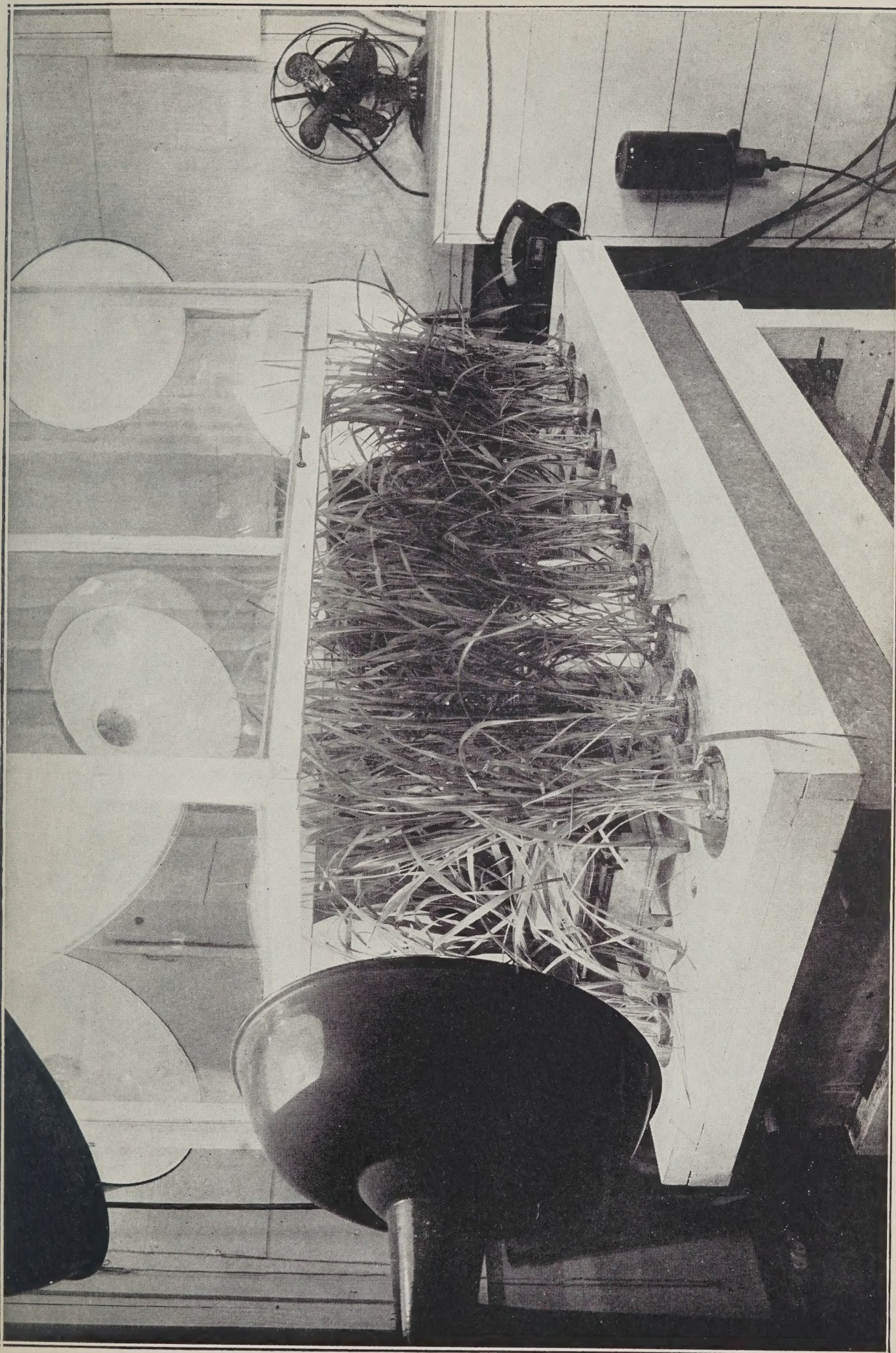
Artificial cultures have proved the following important facts. Although the chemical analysis of the ash of plants shows all the various elements which occur in natural soils, most of these substances have no physiological importance. Only two non-metals—sulphur and phosphorus—and four metals—potassium, magnesium, calcium and iron—are absolutely necessary for seed-plants. This can be demonstrated clearly by omitting this or that element from the nutrient solution. If the absent element is not absolutely essential, the plant develops normally when it is omitted. When one of the foregoing six elements is lacking, the plant grows only to the extent that there was a small supply of the particular element in the seed. The presence of all six essential elements of the ash gives just as large a crop as that on a very fertile soil; this means that the plant has all that it needs for its normal nutrition. How the plant develops poorly in the absence of an essential element is shown in Fig. 34 which represents two

^w The modern tendency is to avoid all dependence on climatic factors and the season of the year and to grow the plants in an environment in which every factor is perfectly controlled. An apparatus for this purpose is shown in Fig. 32 and was designed and first used by A. R. Davis and D. R. Hoagland who describe it in *Plant Physiol.* 3: 277-292. 1928. The apparatus is built in units and the several parts control the light intensity and duration, the temperature, humidity and the culture solutions. Each unit costs about \$500 to build and \$2.50 per day to operate. The original paper must be consulted for the full details of its construction and use.—Ed.

¹ Mazé, P. *Ann. Inst. Pasteur.* 14: 26. 1900.

² Mazé, P. *Ibid.* 25: 705. 1911; Grafe. *Sitzungsber. Akad. Wien, Math-natur. Kl.* Vol. 115: 1906; Vol. 118. 1909; Hutchinson and Miller. *Centralbl. f. Bakt. (II)* 30: 513. 1911; Schulow. *Investigations in the field of the physiology of nutrition by the use of individual, sterilized cultures.* 1913 (Russian); Combes, R. *Compt. rend.* 154: 891. 1913; Petrow, G. G. *The nitrogen assimilation of the seed-plants in the light and in darkness*, p. 10. 1917 (Russian); Bobko, E. *Tagebuch d. I Kongr. d. russ. Bot.* p. 34. 1921 (Russian).

³ Schulow. *loc. cit.* P. 119; Petrow, G. G. *loc. cit.* P. 193.



specimens of buckwheat; one plant received all six essential elements but the other lacked potassium. Such clean-cut results can be obtained in scarcely any other field of plant physiology. Incidentally, buckwheat gives larger yields in a normal nutrient solution which contains the six elements of the ash essential for plants, than can be grown under natural conditions. The dry weight of the plant grown can be 4,768 times the weight of the seed.¹ Oats have given an increase in weight of the ratio of 2359 to 1.² A clearer illustration of the synthesis of organic substances by green plants on pure mineral nutrients can hardly be found. Whole trees have been grown in water cultures for several years.

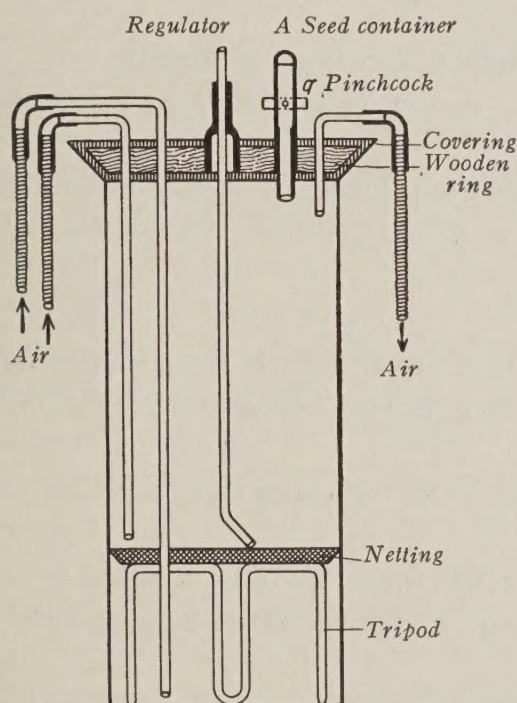


FIG. 33.—Apparatus for pure cultures of seed-plants. Explanation in the text. (After G. G. Petrow.)



FIG. 34.—Buckwheat in water cultures. I. Normal solution. II. Solution without potassium. (After Nobbe.)

Recently it has been found that, aside from these six elements, small (often not indicated by analyses) quantities of manganese, zinc, cerium and silicon are necessary for seed-plants (maize); furthermore, boron, aluminium, bromine, iodine and fluorine are said to be important.³ Since here it is a question of minimum quantities, and a whole series of accurate investigations have already given the opposite results, these new statements need to be confirmed.⁴ It is very possible that small amounts of some non-essential elements act as catalysts to growth. This hypothesis is confirmed by growth experiments with manga-

¹ Nobbe. Landwirt. Versuchs-Stationen. 10: 1 and 94. 1868.

² Wolff. Ibid. 10: 351. 1868.

³ Mazé. Compt. rend. 160: 211. 1915; Idem. Ann. Inst. Pasteur. 33: 147. 1919.

⁴ The solutions used in these experiments were, on the whole, very concentrated and there may have been injurious secondary effects. [See below for further references to other work along these lines.]

nese, chromium, uranium and copper;¹ even sodium salts often increase the crop.²

Various mineral solutions have been suggested for these water and sand cultures. The old solution of Hellriegel contains the following amounts of salt per liter of water or kilogram of sand:

KH_2PO_4 0.136 g.; $\text{Ca}(\text{NO}_3)_2$ 0.492 g.; MgSO_4 0.06 g.;
KCl 0.075 g. and FeCl_3 0.025 g.

(In the molecular ratios of 1:3:1:0.5: trace)

Knop's solution³ has the following composition:

Per liter of water use 1 g. of $\text{Ca}(\text{NO}_3)_2$, 0.25 g. of MgSO_4 ,
0.25 g. of K_2HPO_4 , and a trace of FeCl_3 .

A solution is often used in which the phosphorus occurs in the form of the slightly soluble calcium phosphate, as in natural soils. Thus Crone's solution⁴ has the following composition:

Per liter of water use 1 g. of KNO_3 , 0.5 g. of MgSO_4 , 0.5 g. of $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$,
0.25 g. of $\text{Ca}_3(\text{PO}_4)_2$ and 0.25 g. of $\text{Fe}_3(\text{PO}_4)_2$.

Prianischnikow's solution⁵ contains the following salts per liter of water:

NH_4NO_3 0.240 g.; $\text{CaHPO}_4 + 2\text{H}_2\text{O}$ 0.172 g.; KCl 0.150 g.;
 MgSO_4 0.060 g.; $\text{CaSO}_4 + 2\text{H}_2\text{O}$ 0.334 g.; FeCl_3 0.025 g.

This mixture is used only for sand cultures and is recommended for plants which are sensitive to an acid reaction. Still other nutrient solutions have been proposed.^x Thorough tests indicate that a modified Crone's solution is the most suitable nutrient mixture for most plants. In this solution, all the phosphorus is in the form of $\text{Ca}_3(\text{PO}_4)_2$ and iron is added in the form of the sulphate.⁶ The finest yields of various kinds of plants have been obtained on the foregoing simple nutrient solutions. Under the conditions existing in water cultures, the salts are completely ionized so that it really does not matter to which acids the bases used in the solution were united. There is a recent remarkable state-

¹ Tschirikow, T. Aus den Resultaten der Vegetationsversuche und Laboratoriumsarbeiten von Prof. Prianischnikow. 8: 348. 1913 (Russian).

² Jakuschkin, J. *Ibid.* 8: 315. 1913.

³ Knop. Landwirt. Versuchs-Stationen. 30: 293. 1884.

⁴ Crone, V. v. Diss. Bonn. 1904; *Idem.* Bot. Zeit. 62: 122. 1904.

⁵ Arnold, M. Aus den Resultaten der Vegetationsversuche und Laboratoriumsarbeiten von Prof. Prianischnikow. 8: 7. 1913 (Russian).

^x For other solutions see Raber, O. Principles of Plant Physiology. P. 97. 1928 and Palladin. Plant Physiology. 3rd Amer. Ed. P. 83-84. 1926.—*Ed.*

⁶ Arnold, M. *loc. cit.*; Jakuschkin, J. *Ibid.* 10: 258. 1916 (Russian); Stolhane, A. *Ibid.* 10: 289. 1916 (Russian).

ment that plants absorb their ash constituents better if grown alternately on mineral solutions and pure water.¹

Pasteur² was the first to prove the need of the ash constituents for micro-organisms. Pasteur's student, Raulin,³ performed very accurate experiments on the mineral nutrition of the mold *Aspergillus niger*. He showed that the utilization of the organic foods, especially cane sugar, by the fungus depends on the mineral composition of the nutrient solution. With the optimum proportions of the ash constituents, the fungus *Aspergillus niger* showed an enormous development. Raulin's original solution had the following composition. To 1500 cc. of water add

Cane sugar.....	70 g.	NH ₄ NO ₃	4 g.
NH ₄ H ₂ PO ₄	0.6 g.	MgSO ₄	0.4 g.
K ₂ CO ₃	0.6 g.	(NH ₄) ₂ SO ₄	0.25 g.
ZnSO ₄	0.07 g.	FeSO ₄	0.07 g.

Some of the substances in this solution were not direct nutrients for the fungus but served to balance the effects of ions on the protoplasm and as stimulating factors. Raulin added 4 g. of tartaric acid to his solution to keep the reaction acid but this precaution is superfluous for pure cultures. It also appeared later that the mineral composition of the solution could be simplified considerably. For example, experience shows that the following simple solution may be recommended for *Aspergillus niger*. To each liter of solution add 3 g. of NH₄NO₃, 1 g. of KH₂PO₄, 1 g. of MgSO₄, 0.01 g. of ZnSO₄ and 0.01 g. of FeSO₄.

Thus it has been established that molds can grow on a nutrient solution which contains not six but five elements of ash—phosphorus, sulphur, potassium, magnesium and iron. Calcium is entirely unnecessary for fungi and for all lower plants without chlorophyll and zinc is only a catalyst of their growth. The powerful, growth-promoting effect of zinc ions on the protoplasm of *Aspergillus niger* is specific and cannot be replaced by the effect of another element.⁴ That calcium is not essential to the lower organisms has been confirmed by a series of investigations by various workers.⁵ It is therefore probable that the rôle of calcium in seed-plants has some connection with the photosynthetic nutrition of these plants.^y

¹ Breaseale, J. F. Jour. Agr. Res. 24: 41. 1923. [This method is a slight modification of that of Gericke, W. F. Science. 56: 421-422. 1922 in which single-salt solutions are used in rotation, the roots of the plants being left for a day or a few days in each of the solutions. The method is still being tested further.]

² Pasteur, L. Ann. de chim. et de physique (3) 58: 388. 1860.

³ Raulin. Ann. Sci. Nat. (5) 2: 224. 1869; Idem. Compt. rend. 56: 229. 1870.

⁴ Javillier. Bull. Soc. Biol. 1: 55. 1914; Butkewitsch, W. Biochem. Zeitschr. 132: 556. 1922.

⁵ Winogradsky, S. Arb. d. St. Petersburger Naturforscherges. 14: 132. 1884; Loew, O. Flora. 75: 368. 1892; Molisch. Sitzungsber. Akad. Wien, Math-natur. Kl. 103: 554. 1894; Benecke. Ber. bot. Ges. 12: 105. 1894; Idem. Jahrb. wiss. Bot. 28: 487. 1896; Idem. Bot. Zeit. 54: 97. 1896; 56: 83. 1898; 61: 19. 1903; 62(II): 113. 1904; 65(I): 1. 1907; Wehmer, C. Ber. bot. Ges. 14: 257. 1895.

^y In addition to the points to be brought out later in connection with the rôle of calcium, there is the well established fact that calcium forms calcium pectate as one of the chief constituents of the middle lamella. The situation was summarized by R. H. True in Science. 55: 1-6. 1922. The other principal rôle of calcium that was brought out by True was the function of calcium in making other elements physiologically available. See also W. Mevius. Jahrb. wiss. Bot. 66: 183-253. 1927 in which the relation of the calcium ion to the growth of roots is considered at length.—Ed.

The algae seem to occupy a middle position between seed-plants and the plants without chlorophyll. Some simple forms are said to live without calcium¹ but *Spirogyra*, *Zygnema* and *Mesocarpus* die from lack of it;² hence they require the same mineral nutrition as seed-plants. Calcium is also necessary for the *Cyanophyceae*.³

6. THE PHYSIOLOGICAL IMPORTANCE OF THE SINGLE ELEMENTS AND THEIR COMPOUNDS IN PLANTS

The general viewpoint has just been explained by which to estimate the physiological importance of mineral substances. The old notions that some mineral elements "serve to make foliage," etc. were discarded long ago; in the main they were not explanations of the physiology.

However, it must be borne in mind that each ash element in plants plays its specific rôle which is not sufficiently described by the general notions of hormones, catalysts, antagonistic, etc., if these concepts are not reduced to concrete cases for each element. Unfortunately the special physiological chemistry of the individual ash elements is still incompletely worked out. Thus we do not yet know in all cases in which compounds each ash element occurs in plants and what changes these compounds undergo in living cells. The important specific significance of each element appears in that the essential ash constituents cannot be replaced by others. Attempts have been made to replace potassium by sodium, calcium, lithium, rubidium and caesium for the lower plants⁴ as well as for the higher ones.⁵ The results have been wholly negative. Equally unsuccessful were attempts to replace magnesium by beryllium and other metals.⁶ Likewise calcium, which is required only by green plants, can be replaced neither by excess of magnesium nor by strontium or barium.⁷ Manganese, cobalt and nickel⁸ have been tried in place of iron, arsenic in place of phosphorus⁹ and selenium in place of sulphur.¹⁰ All these experiments proved conclusively the absolute *individual* requirement for these elements. In considering the individual elements we begin with the metalloids which play a different rôle than metals. For the synthesis of the most important protoplasmic colloids—proteins and lecithins—sulphur and phosphorus are just as essential as carbon and nitrogen. For this reason the rôle of phosphorus and sulphur is relatively clear.

¹ Molisch, H. Sitzungsber. Akad. Wien, Math-natur. Kl. I. 104: 783. 1895; Benecke. *loc. cit.*

² Bokorny. Bot. Centralbl. 62: 1. 1895; Benecke. *loc. cit.*; Klebs, G. Bedingungen der Fortpflanzung bei einigen Pilzen und Algen. 1896.

³ Maertens. Cohns Beitr. Biol. d. Pflanzen. 12: 439. 1915.

⁴ Winogradsky, S. Arb. Petersburger Naturforscherges. 14: 132. 1884; Benecke. *loc. cit.*; Molisch. Sitzungsber. Akad. Wien. 15(I): 636. 1896.

⁵ Wolff. Landwirt. Versuch-Stationen. 10: 349. 1868; Nobbe. *Ibid.* 13: 399. 1871; Loew, O. *Ibid.* 21: 389. 1878.

⁶ Benecke. *loc. cit.*

⁷ Benecke. *loc. cit.*; Knop. Landwirt. Versuchs-Stationen. 8: 143. 1866.

⁸ Birner und Lucanus. *Ibid.* 8: 140. 1866; Wagner. *Ibid.* 13: 72. 1871; Knop. Kreislauf des Stoffes, p. 614. 1868; Totttingham and Black. Plant World. 19: 359. 1916.

⁹ Molisch. *loc. cit.*; Stoklasa, J. Zeitschr. f. landwirt. Versuchswesen Österreichs, p. 154. 1898.

¹⁰ Cameron. Proc. Roy. Soc. Dublin, p. 231. 1879.

Sulphur.—Water cultures prove with certainty that the seed-plants are able to use sulphur only in the form of sulphates. Sulphites and thiocyanates are useless for the nutrition of seed-plants.¹ The organic sulpho acids are indeed assimilated but apparently only after the inorganic sulphuric acid is split off. For the fungi, sulphites and thiosulphates can be used as a source of sulphur to a certain extent.²

It is the more remarkable that sulphur can be assimilated only in the form of its highest oxide because sulphates are completely reduced in plants. According to present ideas, sulphur is never found in proteins in any union with oxygen. Moreover in the *Cruciferae* there are mustard oils of the general structure $R-N=C=S$, such as allyl mustard oil, and also leek oil of the general structure $R-S-R$, $R-S-S-R$ or $R-S-S-S-R$.³ In all these compounds there is no sulphur linked to oxygen. Unfortunately there have been no experiments carried out to capture the intermediate products between oxidized sulphur and the foregoing organic compounds. Thus the chemical side of the assimilation of sulphur is entirely unknown. Hydrogen sulphide must be removed from the foregoing derivatives of sulphur during mineralization. This is done by the sulphur bacteria which again oxidize the hydrogen sulphide and complete the cycle of sulphur in nature (p. 155 ff).²

Phosphorus is likewise absorbed by plants only in the form of ortho phosphates. Among the organic compounds of phosphorus, phytin is assimilated but lecithin is not, by pure cultures of seed-plants.⁴ Unlike the sulphates, phosphates undergo no great metamorphosis in the plant body. In proteins the group containing phosphorus is the residue of ortho or meta phosphoric acid. Phosphorus is held in lecithins in the form of the glycerine ester of ortho phosphoric acid. In alcoholic fermentation we find hexose diphosphoric acid (Chap. VIII). Some writers think that such esters of sugars with phosphoric acid are generally common in plants.⁵ In recent years the statement has been made that starch also contains phosphorus⁶ but it must be considered that it is hardly possible to separate starch from the parts of the plastids which contain phosphorus. In any case, the ease with which the phosphoric esters of starch and simple sugars are formed deserves attention.⁷

Perhaps phytin or inosite phosphoric acid is to be regarded as the first product of the assimilation of phosphorus;⁸ phytin represents the union of one

¹ Fittbogen. Landwirt. Jahrb. 13: 755. 1884; Peterson. Jour. Amer. Chem. Soc. 36: 1290. 1914; Tottingham. Jour. Biol. Chem. 36: 429. 1918; and many others.

² Kossowicz, A. und Loew. Zeitschr. f. Gärungsphysiol. 2: 78 and 87. 1912.

³ These substances also occur in species of *Allium*.

⁴ For recent discussions of the relation of sulphur to soil fertility see Adams, H. R. Soil Sci. 18: 111. 1924 and Woodward, J. Bot. Gaz. 73: 81. 1922.

⁵ Schulow, J. loc. cit. P. 157.

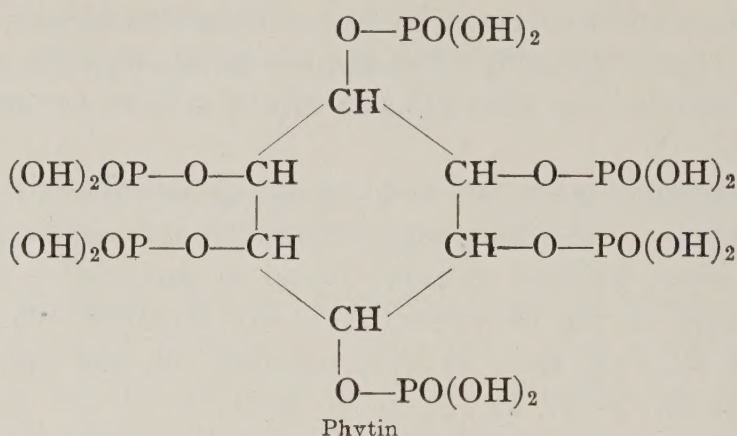
⁶ Nemec, A. und F. Duchon. Biochem. Zeitschr. 119: 173. 1921.

⁷ Thomas. Biochem. Bull. 3: 403. 1914; Kerb, J. Biochem. Zeitschr. 100: 3. 1919.

⁸ Neuberg, C. und Pollak. Biochem. Zeitschr. 23: 515. 1910; 26: 514. 1910; Neuberg. Ibid. 24: 430. 1910; Neuberg und Kretschmer. Ibid. 36: 5. 1911.

⁸ Posternak. S. Compt. rend. 137: 202, 337 and 439. 1903; 140: 323. 1905; 166: 138. 1918; Neuberg und Brahn. Biochem. Zeitschr. 5: 443. 1907; Neuberg. Ibid. 9: 557. 1908; 16: 406. 1909; Winterstein. Zeitschr. f. physiol. Chem. 58: 118. 1907.

molecule of the cyclic, hexavalent alcohol inosite $(\text{CHOH})_6$ with 6 molecules of phosphoric acid.¹



Although the formula of phytin suggests a photochemical formation of this substance, the synthesis of phytin in the light has not been demonstrated.² In the globoids of aleurone grains of various plants, on the contrary, we find a considerable store of phytin.³

The outstanding importance of phosphorus for the organism lies in its presence in the most important protoplasmic colloids and its important rôle in alcoholic fermentation in the form of the phosphoric ester of sugar; the latter is the basis of the processes which release energy in plants.

The transformations of phosphorus in the plant take place chiefly during the growth of organs and the increase in the quantity of protoplasm, which is the time when the proteins containing phosphorus—nucleoproteids and lipoids—are being made.^a During the ripening of seeds, a considerable amount of phosphorus enters the seed in the form of inorganic phosphates and phytin and there are formed all the compounds of phosphorus. These are the facts concerning the transformations and movements of phosphorus compounds on the basis of thorough investigations by Zaleski.⁴ Seed germination is accompanied by a release of considerable amounts of inorganic phosphate.⁵ By the use of microchemical reactions, it is possible to bring out the various details of the localization of phosphorus in different parts of plants.⁶

Information concerning the proteins containing phosphorus and the lipoids will be found in the next two chapters.

Our knowledge of the metals is defective and incomplete.

¹ Posternak, S. *Compt. rend.* 166: 138. 1918.

² Iwanoff, L. The transformations of phosphorus in the plant in connection with the metamorphosis of proteins. 1905 (Russian); Zaleski, W. The transformations and the rôle of phosphorus compounds in plants. 1912 (Russian); Fritsch, R. *Zeitschr. f. physiol. Chem.* 107: 165. 1919.

³ Palladin, W. *Zeitschr. f. Biol.* 31: 191. 1895.

^a This is the basis of the peculiar need for phosphorus at two points in the life history of a seed-plant. The first comes during the first few weeks of the growth of the plant from the seed. The second is the time of formation and maturation of the fruits and seeds. These periods have been observed by several workers. For recent paper which also refers to earlier work see Brenchley, W. E. *Ann. Bot.* 43: 89–110. 1929. The marked use of phosphates during the early weeks of growth was first noted by Gericke (*Science*. 60: 297–298. 1924; *Bot. Gaz.* 80: 410–425. 1925).—Ed.

⁴ Zaleski, W. *loc. cit.*

⁵ Iwanoff, L. *loc. cit.*

⁶ Idem. *Jahrb. wiss. Bot.* 36: 355. 1901.

Potassium seems to play a prominent rôle in the protoplasm. Young active organs, such as meristem, buds, young leaves and living tissues of the bark, are especially rich in potassium. In them the potassium often forms over 50% of the total ash, as shown by numerous analyses of ash.¹ A potassium content of less than 25% is exceptional and occurs in plants with an abnormal composition of ash, in which there is a large deposit of sodium chloride, lime or silica. These substances have no general physiological significance and they accumulate as the result of special ecological conditions for the plants concerned; this will be considered later. Old organs with little protoplasm always contain small amounts of potassium; it passes from them into young parts that are rich in protoplasm. The potassium content is always very high in the ashes of the lower plants without chlorophyll and it even amounts to as much as 60% of the total ash.²

It is remarkable that seed-plants absorb the potassium from the soil chiefly during the early stages of their development.³

The latest studies have proved that all the potassium occurs in plants in the form of ions and there are no complex compounds of potassium.⁴ This shows that its rôle in the protoplasm is very peculiar. Potassium may possibly serve as a source of radioactive forces in the plant.

It is very interesting that potassium is entirely lacking in the cell nucleus,^b the chloroplasts and the cell wall.⁵ This is a new confirmation of its specific rôle. It is said to be important in the synthesis of carbohydrates⁶ and of proteins.⁷ The absence of potassium prevents the development of both higher and lower plants.^c

Magnesium is contained in plant ash in much smaller amounts than potassium but it is also essential for both higher and lower plants.^d Most of the magnesium is held in the seeds. One complex compound of magnesium—chlorophyll—we already know about. In considering the biochemical functions of chlorophyll, we ran into a series of facts and hypotheses which explain the

¹ Wolff. Aschenanalysen. Bd. 1. 1871; Bd. 2. 1880.

² Wolff. *loc. cit.*; Sokolow, N. Analyses of edible fungi. Arb. agrikulturchem. Laborat. d. Forstinstituts. 1873 (Russian).

³ Jegorow, M. Problems in the mineral nutrition of plants. 1923 (Russian); [Bartholomew, R. P. and G. Janssen. Jour. Amer. Soc. Agron. 21: 751-765. 1929].

⁴ Kostytschew, S. und P. Eliasberg. Zeitschr. f. physiol. Chem. 111: 228. 1920.

^b This is contradictory to the idea that is frequently expressed that potassium is essential for cell division. If the potassium does not locate in the nucleus at least it appears to localize in meristematic tissue, according to Janssen, G. and R. P. Bartholomew. Jour. Agr. Res. 38: 447-465. 1929.—Ed.

⁵ Weevers, Th. Recueil des travaux bot. Néerland. 8: 289. 1911.

⁶ Stoklasa, J. Biochem. Zeitschr. 73: 107. 1916; 82: 310. 1917.

⁷ Weevers, Th. *Ibid.* 78: 354. 1917. [With respect to the use of potassium in connection with the synthesis of both proteins and carbohydrates, Janssen and Bartholomew (*loc. cit.*) suggest that it may be associated with condensations and polymerizations of these substances. As the result of his work with potassium and the functions of leaves, James, W. O. Ann. Bot. 44: 173-198. 1930 concludes that there is an "increase of catalytic activity, leading to greater efficiency" in the action of the leaf in the manufacture of carbohydrates.]

^c For a study of the action of potassium starvation and subsequent recovery, see Smith, T. O. and O. Butler. Ann. Bot. 35: 189-225. 1921.—Ed.

^d In the second of two papers on this subject, Canals (Bull. Soc. Chim. Biol. 11: 14-45. 1929) reviews the various possible functions of magnesium. In addition to the rôle of magnesium in chlorophyll, it seems to act as a carrier of phosphates in that it is generally found along with phosphates, especially in seeds. Canals emphasizes the catalytic action of magnesium in connection with enzyme action and reports new experiments in support of this view.—Ed.

great importance of magnesium in complexes for the characteristic properties of the green pigments themselves as well as for photosynthesis.

It is quite possible that the complex compounds of magnesium also play a prominent rôle in physiological syntheses for organisms without chlorophyll. Unfortunately this important question, like many other problems of biochemical reactions which depend on mineral substances, has not even been studied. Magnesium salts which are not balanced [by antagonistic cations such as K, Na and Ca] are toxic to plants.¹ The seed-plants absorb magnesium from the soil at the same rate throughout the period of growth.²

Calcium is contained in large quantities in some plants but in others there is very little. It has been mentioned above that calcium is required only by the plants with chlorophyll but there it cannot be replaced by an increased supply of magnesium. The plants suffer from "lime hunger." Since life in general does not require calcium, it would be of particular interest to discover the *specific* rôle of calcium in green plants. Such an investigation must, of course, be biochemical and not teleological-biological in nature. All the calcium can be removed from dried plant material by means of a triple extraction with water, dilute acetic acid and dilute hydrochloric acid.³ The aqueous and the hydrochloric acid extracts contain only simple calcium salts. In the acetic acid extract there is the calcium that is held in the plant by adsorption.⁴ This can also be extracted by neutral salt solutions and so is probably not held by organic unions.

Some investigators think that the rôle of calcium in seed-plants is very simple; it is said to be connected with the regeneration of proteins from the nitrogen of amino acids and asparagine.⁵ When the framework of amino acids is being formed from sugar, the by-product is oxalic acid which is a powerful protoplasmic poison. Calcium neutralizes the oxalic acid by forming with it an insoluble, harmless compound. However, the situation seems to be more complicated than this and calcium must be used in ways other than the neutralization of organic acids.⁶ It is an interesting point that calcium is the only ash element which has a constant ratio to nitrogen.⁷

There must be an equilibrium between calcium and magnesium in the nutrient or soil solution, else the plants will show various pathological symptoms or they may even die.⁸ This antagonism between the two divalent metals applies to all plants.

¹ Coupin, H. Rev. gén. bot. 32: 19. 1920; Gericke, W. F. Bot. Gaz. 74: 110. 1922.

² Jegorow. loc. cit.

³ Azo. Bull. Coll. Agr. Tokyo. 5: 239. 1902; and others.

⁴ Kostytschew, S. und V. Berg. Planta. 8: 55. 1929.

⁵ Schimper. Bot. Zeit. 46: 65. 1888; Idem. Flora. 73: 207. 1890; Hilgard. Forsch. d. Geb. d. Agrikulturphys. 10: 185. 1887.

⁶ Palladin, W. Ber. bot. Ges. 9: 229. 1891; Prianschnikow, D. The decomposition of proteins during germination. 1895 (Russian); Grabowsky. Aus den Vegetationsversuchen und Laboratoriumsarbeiten von Prianschnikow. 6: 387. 1911 (Russian); Cranner. Jahrb. wiss. Bot. 53: 536. 1914.

⁷ Parker, F. W. and E. Truog. Soil Science. 10: 49. 1920.

⁸ Azo. Bull. Coll. Agr. Tokyo. 4: 361. 1902; 6: 97. 1904; Furuta. Ibid. 6: 371. 1904; Loew, O. Ibid. 4: 381. 1902; Sorokin, H. and Sommer. Amer. Jour. Bot. 16: 23. 1929; Pollack, J. J. Gen. Physiol. 11: 539. 1928.

Iron is usually present in the smallest amounts of all the elements in the ash but in spite of this it is absolutely essential for all plants. It occurs chiefly in young organs which are rich in protoplasm.¹ Plants can absorb it not only in the form of ions but also as a part of complex compounds. Ferrous and ferric salts are assimilated equally well.² Iron cannot be detected in plants by the usual reactions for its ions,³ so it is supposed that it is present in the living tissues in the form of complex organic compounds.⁴ Some nucleins and lipoids containing iron have been prepared and studied.⁵ It has been shown that iron is very important in the processes of oxidation and reduction and its organic derivatives frequently play an important rôle in biological oxidation.⁶ It is uncertain whether this is the only significance of iron in plants but its functions are very specific in any case. In green plants chlorosis is a particularly characteristic pathological phenomenon caused [in some cases] by lack of iron. This was discussed in Chap. II. Undoubtedly there are less striking but just as significant effects as the result of lack of iron in plants without chlorophyll.

In addition to the foregoing essential elements of the ash, there are various other elements in plants. The ash of plants grown under natural conditions contains various mineral substances. Among the metals which are not essential for development, some play certain physiological rôles under natural conditions. For example, **aluminium** and **manganese**⁷ favor the germination of mesophytes⁸ which have an abundance of aluminium in damp places and are poor in it on dry places. Xerophytes contain only a small amount of aluminium.⁹ This element seems to act as a catalyst and hormone¹⁰ in the plants which normally contain it. That an unessential ash constituent may have definite effects on the physiological processes, once it enters the plant, is proved by the fact that the red flowers of *Hydrangea hortensis*, for example, take on a blue color in the presence of aluminium.¹¹

¹ Maquenne, L. et C. Cerigelli. Compt. rend. 173: 273. 1921.

² Knop. Ber. Sächs. Ges. 25: 8. 1869; Wagner. Landwirt. Versuchs-Stationen. 13: 74. 1870.

³ Molisch, H. Die Pflanze in ihren Beziehungen zum Eisen. 1892.

⁴ Petit. Compt. rend. 115: 246. 1893; Vol. 116. 1893; Stoklasa, J. Ibid. 127: 232. 188; and many others.

⁵ Petit. loc. cit.; Stoklasa. loc. cit.; Suzuki. Jour. of Agr. Tokyo. 4: 260. 1901; Glikin. Biochem. Zeitschr. 21: 348. 1909.

⁶ Gola, G. Atti Reale Accad. dei Lincei, rendiconto (5) 24: 1239. 1915; 25: 289. 1915; 28: 146. 1919. [See also Warburg, O. Science. 61: 575-582. 1925; Warburg, O. und E. Negelein. Biochem. Zeitschr. 202: 202-228. 1928.]

⁷ For manganese see the detailed work of E. Uspenski. Jour. Exp. Landwirt. 16: 299. 1915 (Russian); Idem. Jour. Mosk. Abteil. d. Russ. Bot. Ges. 1: 65. 1922 (Russian). [Among the papers in English, the recent one by W. B. S. Bishop. (Austral. Jour. Exp. Biol. & Med. Sci. 5: 125-141. 1928) gives a full review of previous ideas concerning manganese and its function in plants. He concludes that Mn is essential but that it must be present in small amounts. It seems to be related to the formation of chlorophyll. It may be an activator or it may act by modifying the absorption of other elements. Other recent papers which tend to show that Mn is an essential element are: McHargue, J. S. Jour. Amer. Chem. Soc. 44: 1592. 1922; Idem. Sugar. 29: 81-83. 1927; Samuel, G. and C. S. Piper. Ann. Appl. Biol. 16: 493-524. 1929.]

⁸ Loew and Honda. Bull. Coll. Agr. Tokyo. 6: 125. 1904; Stoklasa. Biochem. Zeitschr. 91: 137. 1918; however cf. Ehrenberg und Schultze. Jour. f. Landwirt. 64: 37. 1916.

⁹ Stoklasa, Sebor, Zdobnicky, Tysch, Horak, Nemec and Czwack. Biochem. Zeitschr. 88: 392. 1918.

¹⁰ Schedd, O. M. Jour. Ind. Eng. Chem. 6: 660. 1914.

¹¹ Molisch, H. Bot. Zeit. 55: 49. 1897.

Sodium is entirely unnecessary to land plants but it plays an important rôle in the life of marine plants, especially the algae¹ and likewise inhabitants of salty soil, which will be considered later.^e

Zinc is a powerful catalyst of growth processes. Under the influence of zinc ions, the vegetative growth of the fungus *Aspergillus niger* is much stimulated and the formation of conidia is retarded.² Moreover, it has been shown that zinc and cadmium ions have a strong action on some enzymes; for example, the activity of invertase, reductase and peroxidase is very much retarded by the salts of these metals.³ Probably the ions here affect the colloids.^f

Cobalt and **nickel** are found in all plants that are grown on natural soils.⁴

Among the metalloids, **silicon** accumulates in large amounts in the cell walls of many plants but it has been proved by accurate experiments⁵ that even horsetails *Equisetum*, whose ash is often 70% silica, grows well and normally in the absence of silicon. The same applies to the *Gramineae* in the straw of which there is an accumulation of silica.⁶ Large deposits of this substance are also held in the heart wood of some trees;⁷ of course, there can be no question of a physiological significance of silica at this point. On the other hand, Mazé⁸ found a catalytic effect of very small quantities of silicic acid in the development of plants. The crop is said to be increased considerably by the jelly-like silicic acid, particularly when phosphorus is lacking.⁹ On account of the great technical difficulties, it is impossible to keep cultures of horsetails free from silica even in paraffin vessels.¹⁰ Hence it would be hasty to assume that silicon is an unessential ash constituent.

Boron in small quantities is also said to promote the growth of plants.¹¹ Although the halogens have not been found in plant compounds, **chlorine** was

¹ Osterhout, W. J. V. Bot. Gaz. 54: 512. 1912; Richter, O. Sitzungsber. Akad. Wien, Math-natur. Kl. 118: 1337. 1909.

^e Of course, sodium is always present in plant tissues and it may augment potassium in connection with the antagonistic action of the latter.—Ed.

² Raulin. loc. cit.

³ Kostytschew, S. und S. Zubkova. Zeitschr. f. physiol. Chem. 111: 132. 1920; Jour. russ. bot. Ges. 1: 47. 1916; 3: 40. 1918 (Russian); Kostytschew, S. und G. Medwedew. loc. cit.; Smirnow, A. J. Biochem. Zeitschr. 155: 1. 1925.

^f In recent years a few investigators have obtained evidence which seems to show that zinc is an essential element for the growth of green plants. Excellent photographic evidence for sunflower and barley was given by Sommer, A. L. and C. B. Lipman (Pl. Physiol. 1: 231-249. 1926) who quote previous work in support of this point. Miss Sommer has continued the experiments with these and other species (Sommer, A. L. Pl. Physiol. 3: 217-221. 1928) and reports that species from five families require zinc for normal development. The effect of the absence of zinc is not always the same. Wheat and barley die early but sunflower and buckwheat live on with their growth reduced. Legumes show the effect suddenly but not until the flowering stage.—Ed.

⁴ Bertrand, G. et A. M. Mokragnatz. Compt. rend. 175: 458. 1922.

⁵ Uspensky, E. Tagebuch d. l. Kongr. d. russ. Bot. 1921. P. 26 (Russian).

⁶ Knop. Landwirt. Versuchs-Stationen. 2: 185. 1862; 3: 176. 1862; Wolff. Ibid. 10: 292. 1868; Jodin. Compt. rend. 97: 344. 1884.

⁷ Wolff. Landwirt. Versuchs-Stationen. 26: 415. 1884; and others.

⁸ Mazé, P. Compt. rend. 160: 211. 1915; Idem. Ann. Inst. Pasteur. 33: 147. 1919.

⁹ Lemmermann, O. und H. Wiessmann. Zeitschr. f. Pflanzenernährung. 1: 185. 1922. [But Lemmerman, Wiessmann and Sammet (Ibid. 4: 265. 1925) later concluded that the effect of silica is to increase the up-take of phosphorus.]

¹⁰ Uspensky, E. loc. cit.

¹¹ Warrington, K. Ann. Bot. 37: 629. 1923. [Subsequent work by various investigators seems to have established boron as an essential element which is also toxic if present in appreciable amounts. Sommer and Lipman (loc. cit.) presented both statistical and photographic evidence of the need of boron for

at one time thought to be an essential ash constituent. The later growth experiments disproved this erroneous idea¹ but in some cases anomalous growth has been observed in the absence of chlorine. Perhaps this was due to an unbalanced nutrient solution.

Iodine is found in traces everywhere in the dust of the air, in the soil and in waters.² It is therefore quite conceivable that a certain amount of this element can be detected in every plant which is grown on natural soil. There are statements that iodine is normally contained in the cell nucleus and consequently small quantities of it are necessary for all plant life.³ Likewise some investigators state that a small amount of fluorine has a favorable effect on the development of seed-plants.

7. THE ABSORPTION OF ASH CONSTITUENTS BY PLANTS UNDER NATURAL CONDITIONS

The literature of this question is extremely extensive, because it concerns important problems of present-day scientific agriculture. Here we shall mention only the facts which have a purely physiological bearing. The details may be obtained from the special literature of agriculture.⁴

The main point to be emphasized is that the composition of the ash of different plants from the same place is not entirely alike; moreover, the proportion of the ash constituents in all plants is very different from that in the soil itself. Thus there is a selective absorption of the various salts of the soil by the plants. This unequal absorption is probably due to the unequal adsorption of different salts by both the roots and the soil particles, to changes in the permeability of the protoplasm and to the Donnan equilibrium (see Chap. IX).

If quantitative relations are left out of consideration, it is evident that mineral substances dissolved in the soil water might enter the roots of the plants

beans, buckwheat, flax, mustard, cotton and barley. **W. E. Brenchley** and **K. Warington** (*Ann. Bot.* 41: 167-187. 1927) found boron essential for legumes and melon but not for certain cereals. They found that it is removed from the system and stored in the plants. **Sommer** and **Sorokin** (*Pl. Physiol.* 3: 237-260. 1928) found that the absence of boron made for short, thick and stunted roots, suppression of secondary roots and abnormal cell division in *Pisum sativum*. The work of **E. S. Johnston** and **W. H. Dore** (*Ibid.* 4: 31-62. 1929) with tomato plants also supports the conception of boron as an essential element. See also **McMurtrey, J. E.** *Jour. Agr. Res.* 38: 371-380. 1929 for experiments with tobacco plants. In all these cases, only a trace of boron is required and tolerated by plants.—*Ed.*]

¹ In some plants there are only vanishing amounts of chlorine. On this point *cf.* **Balland.** *Jour. de pharmacie et de chim.* 15: 105. 1917; **Pfeiffer** und **Simmermacher.** *Landwirt. Versuchs-Stationen.* 88: 105. 1916.

² **Gautier.** *Compt. rend.* 128: 643 and 1069. 1898; 129: 189. 1899; **Bourget.** *Ibid.* 129: 768. 1899; 130. 1721. 1900; 132. 1364. 1901.

³ **Justus.** *Virchow's Arch. path. Anat. und Physiol.* 170: 501. 1902; Vol. 176. 1904; however this is disputed by later writers: *cf.* **Blum** und **Grützner.** *Zeitschr. f. physiol. Chem.* 91: 392. 1914; **Winterstein.** *Ibid.* 104: 54. 1918; **Bohn.** *Jour. Biol. Chem.* 28: 375. 1917. [For a recent statement of the occurrence and effect of iodine in plants see **Orr** and **Leitch.** *Spec. Rep. Ser. Med. Res. Council Gt. Brit.* No. 123. 1929.]

⁴ In the Russian literature one will find an abundance of experimental evidence about the nutrition of plants from the soil, in **D. Prianischnikow's** annual "Results of Growth Experiments and Laboratory Studies" from his laboratory at the Moscow Agricultural High School. Prianischnikow's school has worked out the technique of growth experiments very completely. [In England some of the best work appears as the result of experiments carried out at the Rothamsted Experimental Station. In United States such experiments are constantly being reported from various state experiment stations while the work being done in the Division of Plant Nutrition at the Univ. of California deserves special mention.]

and they actually do. For this reason there is a *qualitative* relation between the composition of the soil and that of plant ash. The following table gives an idea of the composition of ash (in per cent) of various plants grown on the same spot, as well as of those constituents of the soil of that place that are soluble in hydrochloric acid.¹

Plants	In per cent of total ash								
	K ₂ O	Na ₂ O	CaO	MgO	Fe ₂ O ₃	P ₂ O ₅	SO ₃	SiO ₂	Cl
<i>Galeobdolon luteum</i>	44.1	0.9	14.0	7.5	0.7	9.8	15.5	2.2	6.9
<i>Ranunculus lanuginosus</i> ...	38.8	0.7	14.2	3.8	0.9	11.7	14.0	2.2	17.7
<i>Maianthemum bifolium</i>	55.7		7.9	8.4	1.2	14.7	3.2	1.9	9.0
<i>Ajuga reptans</i>	28.1	7.0	2.1	5.3	1.4	17.1	10.5	2.2	10.7
<i>Vaccinium Myrtillus</i>	28.1	1.8	27.6	12.5	2.9	9.6	5.2	6.6	2.4
Wood soil.....	0.26	0.10	0.47	0.54	2.91	0.17	0.13		

The following table² illustrates the variations in the composition of the ash of fresh water plants:

Plants	In per cent of total ash								
	K ₂ O	Na ₂ O	CaO	MgO	Fe ₂ O ₃	P ₂ O ₅	SO ₃	SiO ₂	Cl
<i>Chara foetida</i>	0.85	0.44	95.35	0.99	0.07	0.54	0.42	1.22	0.16
<i>Typha angustifolia</i>	32.35	10.98	27.90	1.98	0.20	4.93	3.26	0.79	22.73
<i>Stratiotes aloides</i>	45.09	3.88	15.70	20.99	0.56	4.20	5.09	2.65	2.41
<i>Nuphar luteum</i> , young....	35.88	1.90	32.45	6.55	0.31	9.23	2.39	1.04	6.99
<i>Nymphaea alba</i> , old.....	18.51	25.95	24.27	3.43	0.32	3.31	1.56	0.63	23.11
Water (in per cent).....	0.054	0.282	0.533	0.112		0.006	0.072		0.203
Subsoil (sol. in HCl).....	0.017	0.010	0.468	0.017	0.029	0.034	0.024		

The ash of marine plants usually contains much sodium and sulphur, *e.g.*³

Plants	In per cent of total ash								
	K ₂ O	Na ₂ O	CaO	MgO	Fe ₂ O ₃	P ₂ O ₅	SO ₃	SiO ₂	Cl
<i>Fucus vesiculosus</i>	15.23	24.54	9.78	7.16	0.33	1.36	28.16	1.35	15.24
<i>Laminaria digitata</i>	22.40	24.09	11.86	7.44	0.62	2.56	13.26	1.56	17.23
<i>Laminaria saccharina</i>	12.91	21.86	22.08	14.49	1.06	2.12	23.89		0.53

It is interesting that the composition of the ash of parasites often differs clearly from that of the host plants. This also indicates a selective absorption of salts:⁴

¹ Wolff. Aschenanalysen. 1: 137. 1871.

² Wolff. *Ibid.* 1: 132. 1871.

³ Wolff. *Ibid.* 1: 130. 1871.

⁴ Grandeau, H. et A. Bouton. Compt. rend. 84: 129 and 500.

Plants	Total ash per cent	In per cent of total ash								
		P ₂ O ₅	SO ₃	SiO ₂	CaO	MgO	Fe ₂ O ₃	K ₂ O	Na ₂ O	Cl
<i>Populus</i>	3.097	4.77	1.49	5.81	66.47	8.20	2.38	6.56	2.68	1.64
<i>Viscum</i> on <i>Populus</i>	3.461	26.29	2.09	4.79	32.56	9.21	5.40	16.09	2.04	1.47
<i>Robinia</i>	2.063	3.45	0.78	11.77	75.04	2.51	1.88	2.35	0.47	1.73
<i>Viscum</i> on <i>Robinia</i>	2.132	12.02	2.74	6.41	45.39	6.72	2.20	15.90	2.58	2.02
<i>Picea</i>	1.609	7.89	2.50	2.03	67.43	7.12	1.02	8.40	2.03	1.27
<i>Viscum</i> on <i>Picea</i> ...	3.139	13.11	3.53	1.22	27.13	12.19	1.52	30.79	trace	trace

It can be seen from this table that the mistletoe rapidly absorbs from the host plant the most important elements. On the contrary, the large amounts of calcium deposited in the host are taken in much smaller amounts by the parasite. *Orobanche* likewise collects considerable quantities of potassium.¹ As a rule, the ash content of parasites is large in comparison with the green plants. This becomes especially striking when the weak transpiration of parasites is taken into consideration (it is evident that strongly transpiring plants which have taken in a correspondingly large amount of soil water, contain more mineral substances per unit of dry weight than plants with a low rate of transpiration). Etiolated plants with reduced leaves transpire slowly (light is a powerful stimulant of transpiration). For this reason etiolated plants contain much less ash than normal green plants.² It is easy to understand that the more soil solution enters the plant and the more water escapes as vapor, the richer the plant must be in ash.³ For this reason the representatives of a single species of plants grown on different places and under different ecological conditions do not contain equal amounts of ash. In addition it must be borne in mind that the salts of the soil do not arrive in the plant in the same concentration at which they are present in the soil water. The ions K, NO₃ and PO₄ penetrate faster than water and the ions Ca, Mg and SO₄ more slowly than water. For example, KNO₃ is absorbed correspondingly faster than K₂SO₄.³

The content of soluble salts in the soil solution is very low; in young primary soils it is considerably less than in Knop's solution. On such humus-free soils, however, only the pioneers of the plant world—algae, fungi and lichens—can establish themselves. As they die and become decomposed, the soil comes to have more and more humus and becomes suitable for the nutrition of the seed-plants. In soils rich in humus, most of the mineral salts are present in the adsorbed condition. They are adsorbed partly by the soil colloids and partly by the mucilaginous epidermis of root-hairs.⁴ The relative solubility of salts

¹ Zellner. Monatsh. f. Chem. 40: 293. 1919.

² Palladin, W. Ber. bot. Ges. 10: 179. 1892.

³ This statement need be true only up to a certain point. The first experimental evidence which supported it as a general principle was that offered by Schlösing (Compt. rend. 69: 353-356. 1869) but this work has been refuted by several recent studies including those of Hasselbring. Bot. Gaz 57: 72-73. 1914; Muenscher, W. C. Amer. Jour. Bot. 9: 311. 1922; and Loomis, W. E. Plant Physiol. 4: 158-160. 1929.—Ed.

⁴ Hoagland, D. R. Soil Science. 16: 225. 1923.

⁵ Sabinin, D. Tagebuch d. 1. Kongr. russ. Bot. 1921, p. 35.

in water is less important than the relative adsorption of single ions, in explaining the soil nutrition of plants on natural soils. Strongly adsorbed substances can be washed out of soil by water only with difficulty. Phosphates are held firmly in the soil, sulphates less so and nitrates are easily removed. Potassium is adsorbed better than sodium and divalent metals. It requires about 30 liters of water to wash 1 gram of potassium from the soil.¹ According to the adsorptive capacity of the soil, which depends on its colloidal properties, the soluble substances which come from the disintegration of various minerals remain in the soil and increase its fertility.²

However, the plants are also able in some cases to take from the soil substances insoluble in water, because they put various mineral salts into solution by means of acid secretions of roots. The acid reaction of young roots can easily be shown by litmus paper and the following experiment shows that excretions of roots corrode marble. If a polished marble plate is placed in the bottom of a pot in which plants are growing and left for some time, the impressions of the roots and even of root-hairs can be seen on the smooth surface of the marble. The marble was dissolved by the acid excretions where the roots rested on it.³ If plates of various substances, insoluble in water, mixed with gypsum and then set to a solid mass, are placed in soil which is full of roots of plants, it can be experimentally established which of them are dissolved by root excretions.⁴ On the basis of numerous experiments of this kind, Czapek concludes that roots liberate only carbonic acid and occasionally acid phosphate.⁴ This view, which predominated for some time, can hardly hold now because it has turned out that the plants are able to assimilate some substances⁵ which are not attacked by carbonic acid.⁶ When oxygen is lacking, organic carboxylic acids actually enter the soil from the roots.⁷ Of course, the acid reaction of soil brought about by microorganisms can also help to dissolve the mineral substances in the soil.⁸ In view of these considerations, experiments with pure cultures of seed-plants are especially useful in solving the question of the excretions of roots. These experiments have shown that the roots liberate malic acid and other organic substances, such as sugar, into the surrounding medium when plenty of air is available.⁹ On the whole, the view is now becoming more widespread that roots not only absorb various substances from the soil but also excrete some products of their metabolism into the soil.¹⁰ On

¹ Peters. Landwirt. Versuchs-Stationen. 2: 135. 1860.

² The adsorption phenomena in soils are treated thoroughly in textbooks of soil science.

³ Liebig, J. v. Liebig's Ann. d. Chem. 105: 109. 1858; Sachs, J. Bot. Zeit. 18: 117. 1860.

⁴ Czapek, F. Jahrb. wiss. Bot. 29: 321. 1896.

⁵ Goebel. Pflanzen biol. Schilderungen. 2: 211. 1891; Czapek. *loc. cit.*

⁶ Prianischnikow. Ber. bot. Ges. 22: 184. 1904; Kunze. Jahrb. wiss. Bot. 42: 357. 1906; Pfeiffer, Th. und E. Blank. Landwirt. Versuchs-Stationen. 1912; the results of Dojarenko, A. Jour. russ. chem. Ges. Vol. 41. 1909 (Russian) support Czapek's theory, and likewise those of Aberson, J. H. Jahrb. wiss. Bot. 47: 41. 1910; and Chemin. Compt. rend. 173: 1014. 1921.

⁷ Stoklasa, J. und A. Ernest. Jahrb. wiss. Bot. 46: 55. 1909.

⁸ On this point cf. Kröber. Bot. Centralbl. 113: 140. 1909.

⁹ Mazé. Ann. Inst. Pasteur. 25: 706. 1912; Schulow. Investigations in the field of the physiology of nutrition of the higher plants. 1913, p. 185 (Russian); Minina. Mitt. biol. Inst. Univ. Perm. 5: 233. 1927.

¹⁰ Mazé. *loc. cit.*; Schulow. *loc. cit.*; Czapek. *loc. cit.*; with special reference to the passing of mineral substances into the soil: Wilfahrt. Landwirt. Versuchs-Stationen. 63: 1. 1906; Deleano. Inst. de bot.

the basis of these observations, a theory was proposed¹ to explain the phenomenon of soil fatigue which has long been recognized in agricultural practice. This theory claims that in the process of soil fatigue, *i.e.*, reduction of the crop by repeated growth of the same plant on the same piece of ground, an important rôle is played by various products formed in plants and released to the soil through the roots. Exhaustive studies with both artificial cultures and naturally run-out soils show that substances which retard the growth of plants in the case of repeated sowings, are strongly absorbed by powdered charcoal.² These vanish completely when ignited³ but only in part when boiled.

We have already spoken of the theories which ascribe a prominent rôle to both the ectotrophic and endotrophic mycorrhiza, in providing the seed-plants with ash constituents. Actually the lower organisms often have more effect on the soil components that are insoluble in water than is the case in cultures of seed-plants. It is recognized that lichens attack silicates by means of their acids and contribute much to the disintegration of the most durable kinds of rock.⁴ Many fungi are able to grow very well on highly acid nutrients. Consequently they often make holes in large masses of limestone by means of the acids excreted, and decompose marble and bone.⁵ Some algae produce just as much decomposition of limestone.⁶ It can be assumed that the lower organisms very rarely lack ash constituents under natural conditions.

8. CHARACTERISTICS OF THE COMPOSITION OF THE ASH OF SOME PLANTS

Among all the elements of the ash of plants, calcium, sodium and silicon are remarkable in that their content fluctuates widely. Often these elements occur in plant ash only in extremely small quantities but again their content may be very large. Calcium accumulates in large amounts in the needles of Conifers, in the heart wood and in the bark of many trees, and likewise in all organs of plants growing on soils containing much lime. The quantities of calcium in the old wood are larger than all the other elements. In the dead oak bark the calcium amounts to 95% of the total ash. Figures above 70% are normal for the barks of many trees. The calcium is often deposited in the cell walls of dead tissues. In many plants there are special arrangements for getting rid of the surplus calcium that enters with the soil solution. For

de l'univ. de Genève. Vol. 7 and 8. 1907-1908; Greisenegger und Vorbuchner. Öster.-Ungar. Zeitschr. f. Zucker-Ind. 47: 82. 1918; Jegorow. Problems in the mineral nutrition of plants. 1923 (Russian). It has recently been pointed out that selective absorption of separate ions from salts occurs as follows. Another cation or anion is liberated in place of each cation or anion absorbed so that the concentration of hydrogen ions in the outer solution does not change. On this point *cf.* Redfern, G. M. Ann. Bot. 36: 167. 1922. This process will be considered thoroughly in the chapter on the absorption of soil constituents by roots.

¹ Whitney and Cameron. Bur. of Soils Bull. No. 22, p. 23. 1903; Schreiner, O. and H. Reed. *Ibid.* Bull. No. 40; Schreiner, O., H. Reed and J. Skinner. *Ibid.* Bull. No. 47; Prianischnikow. Aus den Resultaten der Vegetationsversuche und Laboratoriumsarbeiten. 8: 421. 1913 (Russian); Periturin. *Ibid.* 8: 448. 1913 (Russian).

² Periturin. *loc. cit.*; Molliard. Rev. gén. bot. 27: 289. 1915.

³ Pouget et Chouchak. Compt. rend. 145: 1200. 1907.

⁴ Bachmann, E. Ber. bot. Ges. 8: 141. 1890; 10: 30. 1892; 22: 101. 1904; 31: 3. 1913; 35: 464. 1917; 36: 528. 1918; and others.

⁵ Lind. Jahrb. wiss. Bot. 32: 603. 1898.

⁶ Nadson, G. The boring algae and their rôle in nature. 1900 (Russian).

example, there are lime druses in the leaves of some plants rich in lime,¹ which excrete a solution of calcium carbonate. As the water evaporates, the lime is left on the leaf as a flaky crust. There is an interesting phenomenon of the "lime haters" which refers to the facts that some plants cannot tolerate a large amount of lime in the soil. Typical lime-hating plants are peat mosses (species of *Sphagnum*) and some other plants which live on peaty soils, likewise some trees such as the chestnut (*Castanea vesca*). *Sphagnum* does not tolerate even 0.03% CaCO_3 .² In the presence of larger amounts of lime, the lime-hating plants assimilate the other mineral substances poorly and often suffer from chlorosis.³ It was once supposed that lime-hating plants can tolerate all but calcium and that they do not suffer in the presence of considerable amounts of Mg. These views are now regarded as superceded. It is now established beyond doubt⁴ that the injurious effect of calcium carbonate is due solely to the alkaline reaction of this salt, since *Sphagnum* can grow only at higher concentrations of hydrogen ions. That calcium is not of itself injurious to *Sphagnum* explains why direct analyses show a rather high content of Ca in the ash of *Sphagnum*.⁵

Sodium accumulates in the form of sodium chloride in large quantities in the halophytes, *i.e.*, inhabitants of soils containing much salt. The capacity to tolerate high concentrations of common salt in the substrate makes it possible for these plants to grow on soils which are not adapted to other plants. Unlike the salts of calcium and silicon, sodium chloride remains in the plant in the dissolved condition, thus producing a considerable osmotic pressure of the cell sap. In this connection, the halophytes have a peculiar structure; the leaf surface is reduced and the plants are fleshy. The halophytes are usually classed, after Schimper,⁶ with the xerophytes—the plants adapted to live in dry places—because it is thought that halophytes are obliged to have a low rate of transpiration in order that too much NaCl may not accumulate in their tissues. The latest objections to this viewpoint⁷ have often been given too little attention. Still it must be borne in mind that the succulents, including the halophytes, form a special group and that they are not a part of xerophytes in the narrow sense. It is known that the halophytes can be grown on the ordinary nutrient solutions and that they lose their specific fleshy structure under these conditions.⁸ To be sure, the latest investigations of Keller⁹ show that *Salicornia herbacea*

¹ Volken. Ber. bot. Ges. 2: 334. 1884; Woronin. Bot. Zeit. 43: 177. 1885; Vuillemin. Ann. Sci. Nat. Bot. (7) 5: 152. 1887; Gardiner, W. Quart. Jour. Micros. Sci. 21: 407. 1881.

² Paul. Ber. bot. Ges. 24: 148. 1906.

³ Roux. Traité des rapports des plantes avec le sol. 1900.

⁴ Korsakowa, M. Arb. Petersburger Naturforscherges; cf. also Mevius, W. Jahrb. wiss. Bot. 60: 147. 1921; Idem. Biochem. Zeitschr. 148: 548. 1924; however cf. Dahm, P. Jahrb. wiss. Bot. 65: 314. 1926.

⁵ Wolff. Aschenanalysen. 1. 135. 1871.

⁶ Schimper, A. Pflanzengeographie auf physiol. Grundlage, vol. 98. 1888.

⁷ Cf. *e.g.*, Fitting, H. Zeitschr. f. Bot. 3: 265. 1911; and others.

⁸ Batalin, A. Bull. Cong. Int. Bot. et Hort. St. Pétersbourg. 1885, p. 219; Lesage. Rev. gén. bot. 2: 54. 1890; Baumgärtel. Sitzungsber. Akad. Wien, Math.-natur. Kl. I. 126: 41. 1917.

⁹ Keller, B. Mitt. landwirt. Inst. Woronesch. Vol. 2. 1916 (Russian); Idem. Anz. d. Versuchsanst. d. Mittelgebietes d. Schwarzerde, Vol. 1. 1921 (Russian). Cf. also Halket. Ann. Bot. 29: 143. 1915; Kolkwitz, R. Ber. bot. Ges. 35: 518. 1917; 36: 636. 1918; 37: 343. 1919.

grows better in the presence of sodium chloride. This halophyte grows so rapidly that at the start there is no increase in concentration of salt in the cell sap; the excess NaCl passes into the newly formed tissue. Moreover, Keller points out that even though the typical structure of *Salicornia* can be produced by concentrated solutions of various salts, sodium chloride still has a more powerful effect than other salts.¹ Thus we seem to be dealing here with a true stimulation of growth and form. Consequently Keller characterizes the halophytes as osmophilous plants.

Silica is deposited in the cell walls of many plants, especially the grasses and horsetails. It was pointed out above (p. 210) that it is possible to grow normal plants without adding silica to the substrate. It is quite possible that silica has no specific physiological meaning² and accumulates especially in plants which absorb it intensively by virtue of their individual natures. The earlier view that silica increased the strength of the grass stem proved to be erroneous. The strength of a stalk of grass is due primarily to its excellent structure and extremely advantageous distribution of the mechanical tissues.³

To summarize the foregoing expositions of the quantitative proportions of the separate elements in the ash of various plants, and of the physiological importance of these elements, it seems that it is not yet possible to state a definite inference at this time. As yet we cannot determine how far the composition of the ash is conditioned by external ecological and internal, physiological conditions.⁴ Probably both factors are important. As for the physiological chemistry of the single elements of the ash, it has begun to develop. The results already obtained emphasize the fact that the importance of the mineral substances must be judged from a different viewpoint than that of the organic substances.

9. MINERAL FERTILIZERS

Agricultural operations take from the soil every year a considerable amount of mineral substances which are drawn away from the fields with the crops. The following table shows the quantity of the several ash constituents that is taken from the soil in kilograms per hectare by the different average crops.

Crop of	K ₂ O	CaO	MgO	P ₂ O ₅	SO ₃	SiO ₂
Winter grains.....	39.2	13.7	8.8	23.5	4.9	105.8
Summer grains.....	49.0	17.6	9.8	19.6	5.9	86.2
Legumes.....	58.8	58.8	15.7	27.4	9.8	9.8
Clover.....	117.5	117.5	41.1	35.3	11.8	19.6
Potatoes.....	105.8	35.3	19.6	33.3	15.7	7.8

It is evident from this table that clover exhausts soil especially rapidly. For just this reason clover must be used carefully to enrich the soil in nitrogen.

¹ Keller, B. *loc. cit.*

² However cf. Mazé, P. *Compt. rend.* 160: 211. 1915; *Idem.* *Ann. Inst. Pasteur.* 33: 147. 1919.

³ Schwendener, S. *Das mechanische Prinzip im anatomischen Bau der Monokotylen.* 1874.

⁴ However, the general picture of the distribution of the single elements in the ash is being interpreted as characteristic of the different groups of plants. Cf. Molisch, H. *Anz. Akad. Wien.* 1920, p. 181; Ziegenspeck. *Ber. bot. Ges.* 40: 78. 1922.

For reasons which were given in the consideration of the question of nitrogen fertilizers, it is clear that fertilizing the soil with manure alone cannot replace the loss of mineral substances. Of course, almost all substances required by plants are, however, held in the soil in insoluble compounds, that is to say, in the rocks from which the particular soil originated. These rocks gradually disintegrate and renew the supply of ash constituents which can be absorbed by the plants and assimilated. Although this disintegration is accelerated by the mechanical working of the soil and by the activities of soil microorganisms, it still proceeds more slowly than the absorption of mineral substances by crop plants. Therefore the land must be allowed to lie fallow in order to increase its fertility. Of course, this method can be used to only a limited extent in intensive farming and mineral fertilizers must be added.

The soil is exhausted most rapidly of phosphorus and potassium and these elements in particular must be added in farming. In addition, fertilizers containing also Mg and sul-

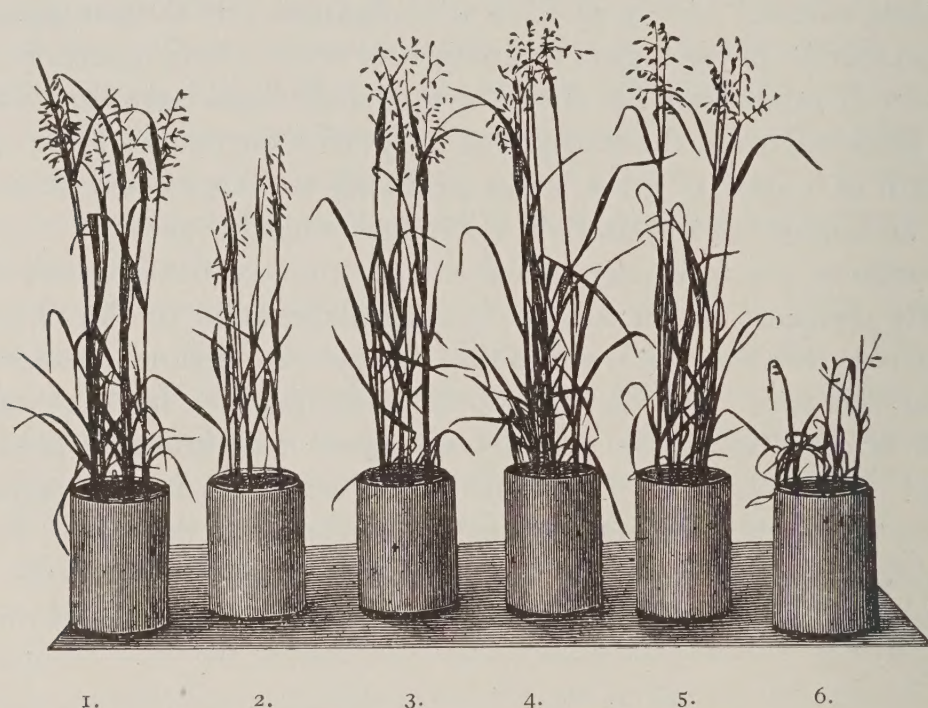


FIG. 35.—The effect of salts of ammonia on the assimilation of phosphorus: 1. $\text{NaNO}_3 + \text{KH}_2\text{PO}_4$. 2. $\text{NaNO}_3 + \text{phosphorite}$. 3. $\frac{3}{4}\text{NaNO}_3 + \frac{1}{4}(\text{NH}_4)_2\text{SO}_4 + \text{phosphorite}$. 4. $\frac{1}{2}\text{NaNO}_3 + \frac{1}{2}(\text{NH}_4)_2\text{SO}_4 + \text{phosphorite}$. 5. $\frac{1}{4}\text{NaNO}_3 + \frac{3}{4}(\text{NH}_4)_2\text{SO}_4 + \text{phosphorite}$. 6. $(\text{NH}_4)_2\text{SO}_4 + \text{phosphorite}$. (After Prianschnikow.)

phates are used to a more limited extent. Kainite $\text{KClMgSO}_4 + 3\text{H}_2\text{O}$ is an outstanding fertilizer by which three elements essential to plants are added to the soil at the same time. Other minerals containing K are also used, likewise potassium chloride from the salt of the ocean.

Particular attention has been given to the question of phosphorus fertilizers on behalf of both practical and scientific agriculture.¹ In agriculture, this fertilizer is often used in the form of phosphorites, *i.e.*, minerals which contain phosphorus as calcium phosphate. In recent years successful use has been made of the so-called Thomas slag meal, a by-product of smelting ores containing phosphorus, in place of phosphorites. This also holds the phosphorus in the form of insoluble calcium phosphate. Not all the phosphorus of this product is available to plants and not equally to all plants. It has been proved that plants are generally able to absorb the compounds of phosphorus which are soluble in acid ammonium citrate.²

¹ Prianschnikow, D. The science of fertilizers. 1922 (Russian).

² König, J. Untersuch. landwirt. wichtiger Stoffe, p. 161. 1898. This method is contested by many investigators: *cf. e.g.*, Dafert und Reitmair. Zeitschr. f. landwirt. Versuchswesen Öst. 3: 589. 1900. A new method is given by O. Arrhenius. Zeitschr. f. Pflanzenernähr. (A) 14: 121. 1929. Another interesting biological method is described by N. Bessonow. La Science du Sol. 7: 1. 1928; Compt. rend. 186: 522. 1928.

Exhaustive studies made by Prianischnikow and his co-workers¹ have explained the complicated relations in the assimilation of phosphorus from the phosphorites by different plants in different cultures. For example, the *Leguminosae* assimilate the phosphorus of phosphorites much better than the *Gramineae*; on fertile soil phosphorite has less effect than on less fruitful soils. Of interesting and practical importance is Prianischnikow's improvement of phosphorus assimilation by the *Gramineae* by means of the simultaneous use of nitrogen fertilizers in the form of physiologically acid ammonium salts (Fig. 35). The acid released by the assimilation of ammonia dissolves the phosphorite. This makes it possible to use a cheap nitrogen fertilizer with success and to insure the use of phosphorite at the same time. For example, the following yields were obtained with oats:²

Phosphorite + NaNO ₃	Phosphorite + $\frac{3}{4}$ NaNO ₃ + $\frac{1}{4}$ (NH ₄) ₂ SO ₄
6.9	22.0
Phosphorite + $\frac{1}{2}$ NaNO ₃ + $\frac{1}{2}$ (NH ₄) ₂ SO ₄	KH ₂ PO ₄ + NaNO ₃
20.5	19.7

In any case, an insoluble phosphorus fertilizer cannot be used on any soil and for any crop without running controls; the results may be negative in many cases. In order to increase the assimilation of phosphorus and to change the fertilizer to a generally useful form, one often prepares the so-called superphosphate, by treating various waste-products of factories and insoluble phosphate materials with sulphuric acid or sodium sulphate.³ This gives a phosphorus fertilizer that is well assimilated.

10. ANALYTICAL METHODS

To prepare the ash, the plant material is burned either with or without the addition of some salt. Another method of combustion is to oxidize the material in a liquid.

Incineration without adding anything is done by careful heating in a platinum dish. The method is not suitable for all determinations because it does not prevent a small loss of some substances, especially sulphur and other metalloids. To determine the substances exactly, the plant material is incinerated with soda and saltpeter. Three volumes of a mixture of 1 part Na₂CO₃ and 2 parts KNO₃ are usually added to one volume of finely divided plant material. Incineration is now being done by the "wet" process. A popular method is that of Neumann,⁴ according to which the plant material is burned by careful heating with a mixture of equal parts concentrated sulphuric and nitric acids. This mixture is added slowly in small portions until dark-brown fumes no longer appear and the solution has become clear. Various other analogous methods have been proposed.⁵ It seems to be still better to mineralize the material by oxidation with hydrogen peroxide in the presence of a ferrous salt⁶ but it is still uncertain as to how valid this method is for general use.

The qualitative and quantitative determinations of the separate elements in the ash are done by the usual methods. Microchemical reactions are often used for qualitative tests in which the characteristic form of the crystals of an insoluble precipitate are observed under the microscope. For example, potassium platonic chloride, calcium sulphate and the like are easily recognized.⁷ This method does not require the separation of the mineral elements from

¹ Numerous papers in the "Resultaten der Vegetationsversuche und Laboratoriumsarbeiten," Vols. 7-10: cf. also Arb. d. Agr. Lab. in St. Petersburg. 1900 (Russian).

² Prianischnikow, D. Anz. d. Moskauer landwirt. Inst. Vol. 7, 1901 (Russian); *Idem.* Ber. bot. Ges. 23: 8. 1905.

³ Kotschekow, W. Resultaten der Vegetationsversuche und Laboratoriumsarbeiten by D. Prianischnikow. 8: 60 and 69. 1913 (Russian); Periturin, F. *Ibid.* 8: 142. 1913 (Russian).

⁴ Neumann, A. Zeitschr. f. physiol. Chem. 37: 115. 1903; 43: 32. 1904; *Idem.* Pflüger's Arch. f. ges. Physiol. for 1905. P. 208.

⁵ Kastle, J. H. Amer. Jour. Physiol. 22: 411. 1898; Friedmann. Zeitschr. f. physiol. Chem. 92: 46. 1914; Duret. Compt. rend. 167: 129. 1918; Greenwald. Jour. Biol. Chem. 37: 439. 1919.

⁶ Mandel, J. A. und C. Neuberg. Biochem. Zeitschr. 71: 196. 1915; Salkowski, E. Zeitschr. f. physiol. Chem. 96: 323. 1916.

⁷ Molisch, H. Mikrochemie der Pflanzen. 1913; Tunmann, O. Pflanzenmikrochemie. 1913; and others [such as Palladin's Plant Physiology, 3rd Amer. Ed., pp. 90-91 and papers cited there].

each other. Moreover, the microchemical tests make it possible to localize the elements in different parts of the plant and even within the cells. Still it must always be considered that the microchemical tests cannot be used for quantitative determinations. In this connection there have been many abuses.

Hamburger¹ proposed an ingenious method for the quantitative determination of potassium ions. Potassium is precipitated with sodium cobaltic nitrite as a double salt, the crystalline precipitate is centrifuged under certain conditions in calibrated capillary tubes and the amount of potassium calculated from the volume of the precipitate. This does away with the need of incinerating the material and extracts can be used directly.

For phosphorus there are numerous methods which provide for determinations of this element in the separate groups of plant compounds. After incinerating, the total amount of phosphorus can be determined best by Neumann's method as follows.² The phosphorus is precipitated with ammonium molybdate, the precipitate dissolved in titrated sodium hydroxide, heated for 15 minutes to drive off the ammonia, and titrated with sulphuric acid. 1 mg. of NaOH used corresponds to 1.268 mg. of P_2O_5 . The phosphorus of lecithin and the other lipoids is measured by its content in alcohol and ether extracts which do not include the inorganic phosphates.³ The water-soluble compounds of phosphorus include inorganic phosphates, phytin, phosphoric esters of sugar and glycerophosphoric acid. The only organic compound of phosphorus in seed-plants is phytin, the amount of which is quite large. Phytin is precipitated with magnesia mixture along with the inorganic phosphates but it can also be separated in the form of the iron salt in the presence of 0.6% HCl, while the inorganic phosphates remain in solution. Therefore phytin is determined as follows in the presence of inorganic phosphates.⁴ After adding HCl to 0.6% and ammonium thiocyanate to 0.03%, titrate with a solution of 0.05% ferric chloride and 0.6% HCl until a red color appears. 1 mg of Fe corresponds to 1.19 mg. of phytin phosphorus. After the total phosphorus in the aqueous extract is determined and the phosphorus precipitated with magnesia mixture is subtracted from it, the result is the phosphorus of the sugar- and glycerophosphoric acids, which are not precipitated by the magnesia.

The magnesium precipitate is dissolved in dilute nitric acid and the inorganic phosphate is precipitated with ammonium molybdate in the presence of ammonium nitrate.⁵ This separates from the aqueous extract: 1. the phytin phosphorus, 2. the phosphorus of glycerophosphoric acid, and 3. the inorganic phosphorus.

The determination of protein phosphorus is very important because it can often be used for quantitative measurements of the proteins of the living protoplasm, *i.e.*, the nucleoproteins, as well as to distinguish these from the reserve proteins. The phosphorus of the nucleoproteins is determined by Plimmer's method.⁶ The material is treated for 2 days with 1% NaOH at 37°C., the nucleoproteins precipitated by neutralizing the liquid with 0.2% HCl and alcohol, and the precipitate incinerated by Neumann's method. Under the influence of the hydroxide, the phosphorus is split from all proteins except the nucleoproteins.

¹ Hamburger, J. *Biochem. Zeitschr.* 71: 415. 1915; 74: 414. 1916. For other new methods for determining potassium see: Meillère, G. *Jour. Phar. et Chim.* (7) 7: 281. 1913; Hill. *Amer. Jour. Sci.* (4) 40: 75. 1915; Rhue. *Jour. Ind. and Eng. Chem.* 10: 429. 1918; Blumenthal. *Ibid.* 9: 753. 1917; Duboux. *Compt. rend.* 159: 320. 1914; Zaleski, L. *Landwirt. Versuchs-Stationen.* 83: 221. 1913; Haff and Schwartz. *Jour. Ind. and Eng. Chem.* 9: 785. 1917; Mitscherlich, E. A. und H. Fischer. *Landwirt. Versuchs-Stationen.* 76: 139. 1911; 78: 75. 1912; Neubauer, H. *Zeitschr. f. analyt. Chem.* 43: 14. 1904; Herzog, A. *Chem. Zeit.* 42: 145. 1918; Baxter. *Jour. Amer. Chem. Soc.* 42: 735. 1920; [Lonstein, I. *Jour. So. Afr. Chem. Inst.* 10: 27. 1917.]

² Neumann, A. *loc. cit.*; for microchemical determination of P: Terada, Y. *Biochem. Zeitschr.* 145: 426. 1924.

³ Schulze, E. und Steiger. *Zeitschr. f. physiol. Chem.* 13: 365. 1889; Schulze und Winterstein. *Handbuch. biochem. Arbeitsmethoden* von Abderhalden. 2: 256. 1909.

⁴ Heubner, W. *Biochem. Zeitschr.* 64: 422. 1914.

⁵ Heubner, W. *Ibid.* 64: 401. 1914.

⁶ Plimmer and Bayliss. *Jour. of Physiol.* 33: 439. 1906; Plimmer and Scott. *Ibid.* 38: 247. 1909; Idem. *Jour. Chem. Soc. (London)* 93: 1699. 1908.

Sulphur can be determined by the aid of the volumetric method described for potassium, in the form of barium sulphate.¹ New methods have also been worked out for the determination of silica.²

Magnesium is determined in the form of the pyrophosphate according to the usual methods. In order to separate it from the alkali metals, it is often precipitated with dimethylamine or guanidine.³ New methods have been proposed recently for the determination of small amounts of magnesium, calcium and iron.⁴

¹ **Hamburger.** Biochem. Zeitschr. **77**: 168. 1916; **Idem.** Zeitschr. f. physiol. Chem. **100**: 221. 1917. For the determination of sulphur see also: **Stevens.** Analyst. **40**: 275. 1915; **Krieger.** Chem. Zeit. **39**: 22. 1915; **Liebesny.** Biochem. Zeitschr. **105**: 43. 1920; **Krieble and Magnum.** Jour. Amer. Chem. Soc. **41**: 1317. 1919; **Winkler.** Zeitschr. f. angew. Chem. **33**: 59. 1920.

² **Salkowski, E.** Zeitschr. f. physiol. Chem. **83**: 143. 1913; **Lenher and Truog.** Jour. Amer. Chem. Soc. **38**: 1050. 1916; **Horvath.** Zeitschr. f. analyt. Chem. **55**: 543. 1916.

³ **Herz, W. und Drucker.** Zeitschr. f. anorg. Chem. **26**: 347. 1901.

⁴ For Mg: **Dienes.** Biochem. Zeitschr. **95**: 131. 1919; **Blasdale, W. C.** Jour. Amer. Chem. Soc. **31**: 917. 1909; **Eisenlohr.** Ber. chem. Ges. **53**: 1476. 1920. For Ca: **Aron, H.** Biochem. Zeitschr. **4**: 268. 1907; **Gutmann, S.** *Ibid.* **58**: 470. 1914; **Blasdale, W. C.** *loc. cit.*; **Denigès.** Compt. rend. **170**: 996. 1920; **Kuzirian.** Jour. Amer. Chem. Soc. **38**: 1996. 1916; **Halversan and Bergeim.** Jour. Biol. Chem. **32**: 159. 1918; **Jansen, W. H.** Zeitschr. f. physiol. Chem. **101**: 176. 1918; **Dienes.** *loc. cit.* For Fe: **Salkowski, E.** *loc. cit.*; **Berg.** Chem. Zeit. **41**: 50. 1917; **Gonnermann.** Biochem. Zeitschr. **95**: 286. 1919; **Mawas.** Compt. rend. soc. de biol. **82**: 78. 1919; **Mathieu.** Bull. Assoc. Chim. Sucr. **37**: 205. 1919; **Willstätter.** Ber. chem. Ges. **53**: 1152.

CHAPTER VI

CARBOHYDRATES AND PROTEINS: THE TRANSFORMATIONS OF THESE SUBSTANCES IN THE PLANT

1. THE GENERAL IDEA OF THE CYCLE OF PRIMARY SUBSTANCES

The carbohydrates and proteins are direct products of photosynthesis and we call them *primary* substances of plants. From them all the other organic compounds are made and these new products we call *secondary* substances. Those which differ greatly from the primary substances and never change back to them will be considered in the next chapter. The physiological transformations of the primary substances are extremely important. In the animals the proteins play the chief rôle but in plants the important thing is the cycle of carbohydrates from which proteins and fats are made.

With respect to the particularly important physiological significance of carbohydrates and proteins, their chemical characteristics, including consideration of their most interesting physiological properties, will be given before describing their transformations in plants.¹ In the general manuals of organic chemistry and biochemistry, the pertinent information is now obsolete in certain respects, as the result of the rapid progress of the chemistry of the carbohydrates and proteins.^h

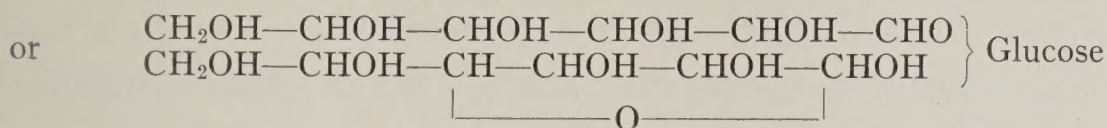
2. THE SIMPLE SUGARS

The term sugar is applied to aliphatic hydroxy aldehydes (aldoses) or hydroxy ketones (ketoses) with unbranched carbon chains, in which there is an aldehyde or a ketone group and whose empirical structure is expressed by the formula $C_mH_{2n}O_n$; in other words they contain 2 atoms of hydrogen to 1 atom of oxygen (with few exceptions). From this comes the term "carbohydrate." The simple sugars are crystalline substances with a sweet taste, and they are very soluble in water, scarcely soluble in alcohol and insoluble in ether. They are called dioses, trioses, tetroses, pentoses, hexoses, etc. according

¹ The most important special literature on carbohydrates and proteins are: **Tollens**. Kurzes Handbuch der Kohlenhydrate. 2nd Ed. 1898; **Lippmann**. Chemie der Zuckerarten. 3rd Ed. 1904; **Abderhalden**. Biochem. Händlexikon, Vol. 2. 1911; Vol. 8. 1914; **Fischer**, E. Untersuchungen über Kohlenhydrate und Fermente. 1909; **Pringsheim**, H. Die Polysaccharide. 2nd Ed. 1923; **Karrer**, P. Einführung in die Chemie der polymeren Kohlenhydrate; **Osborne**, Th. B. The Vegetable Proteins. 1909; **Plimmer**, R. H. A. The Chemical Constitution of the Proteins. 2nd Ed. 1913; **Kestner**, O. Chemie der Eiweisskörper. 4th Ed. 1925; **Fischer**, E. Untersuchungen über Aminosäuren, Polypeptide und Proteins. 1906; **Loeb**, J. Proteins. 1924; **Sørensen**, S. P. L. Proteins. 1925; **Pryde**, J. Recent Advances in Biochemistry. 1926; **Blanchetière**, A. La structure chimique des matières protéiques. 1925; **Edlbacher**, S. Die Strukturchemie der Aminosäuren und Eiweisskörper. 1927; **Kossel**, A. The Protamines and Histones. 1928; **Guggenheim**, M. Die Biogenen Amine. 1924.

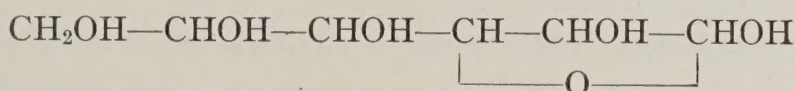
^h This is not true of the very latest texts such as **Gortner**. Outline of Biochemistry. 1929.—Ed.

to the number of carbon atoms in the molecule. The formula is written as follows:

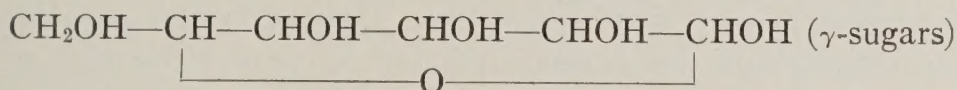


The second formula was introduced because the aldehydic properties of sugars are often present in an almost latent condition. Various facts support this formula.¹ It seems that the first arrangement changes easily to the second and vice versa.

According to the latest information, the foregoing butylene oxide ring often changes to the propylene oxide ring



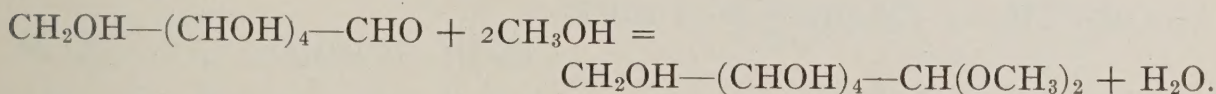
or to the amylen oxide ring



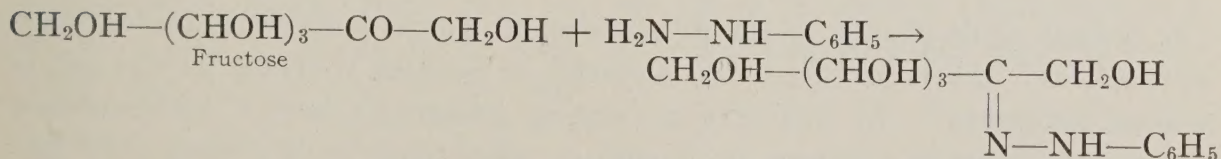
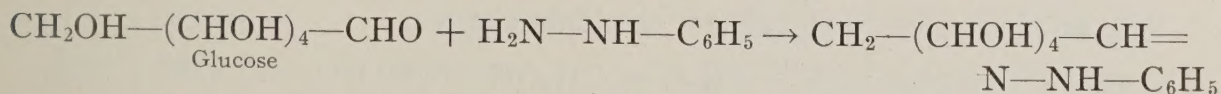
For the most part these changes lower the stability of the molecule.¹

The simple sugars reduce alkaline solutions of copper oxide when boiled; they give acetyl derivatives when warmed with acetic anhydride; with phenyl isocyanate (phenyl urethane) they also form analogous products;² for example, with pentoses the result is $\text{C}_5\text{H}_6\text{O}(\text{O}-\text{CO}-\text{NH}-\text{C}_6\text{H}_5)_4$.

With alcohols the sugars yield acetals, in the presence of HCl according to the general reaction of aldehydes, *e.g.*:



Compounds of sugar with two molecules of phenylhydrazine—the so-called osazones³—are very characteristic. Phenylhydrazine plus a sugar gives at first a soluble hydrazone according to the general reaction of aldehydes and ketones:



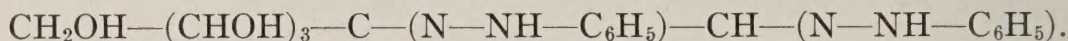
¹ For an account of this point see Charlton, W., W. N. Haworth and S. Peat. Jour. Chem. Soc. (London) 129: 89-101. 1926.—Ed.

² Haworth, W. and J. Mitchell. Jour. Chem. Soc. (London) 123: 301. 1923; Haworth, W. and W. Linnel. *Ibid.* 123: 294. 1923; Irvine, J. *Ibid.* 123: 898. 1923.

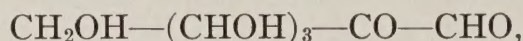
³ Maquenne, B. et Goodwin. Compt. rend. 138: 633. 1904.

³ Fischer, E. Ber. chem. Ges. 17: 579. 1884; 20: 821. 1887; 23: 2117. 1890; 41: 77. 1908.

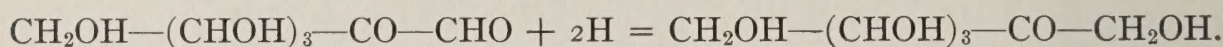
If the heating with phenylhydrazine is continued, there is an oxidation of the hydroxyl groups which are adjacent to the carboxyl groups and a second molecule of phenylhydrazine is added; this forms the osazone:



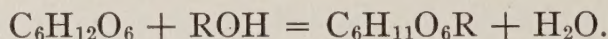
If this formula is compared with the two preceding ones, it will be seen that both aldose and ketose give identical osazones. Boiling with concentrated HCl transforms the osazone into the so-called osone



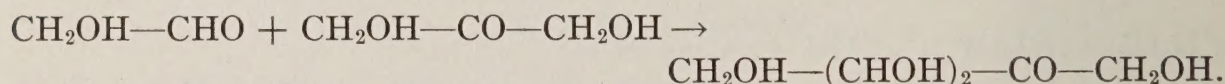
which is precipitated as the lead derivative and can be reduced with zinc dust and acetic acid (of course only to a ketose).



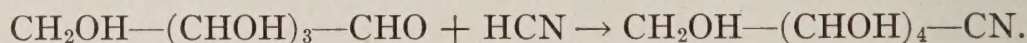
Osazones are yellow, crystalline substances, slightly soluble in water. They are especially well suited to the isolation of the individual sugars, even if the latter are present in very small quantities. Hydroxylamine and sugars form oximes and semicarbazid yields semicarbazone (*cf.* general manuals of chemistry). The action of alcohols in the presence of HCl under certain conditions gives half-acetal-like glucosides,¹ *e.g.*;



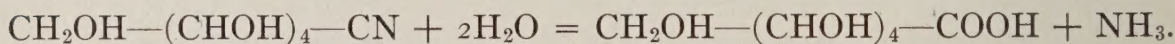
Unlike the typical acetals, sugar unites with one molecule of alcohol. Complicated glucosides are very widely distributed in plants. This will be discussed later. The aldol condensation allows the possibility of obtaining the higher sugars from the lower ones, *e.g.*:



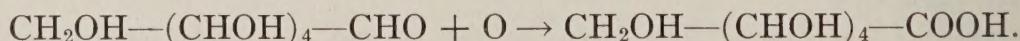
Another way to lengthen the chain, that is of general significance and possibly takes place under natural conditions, occurs as follows.² Acid nitriles are formed by the action of hydrocyanic acid:



The nitriles saponify to acids of the higher series:



The hexone acids change to the intramolecular esters, lactones $\text{C}_6\text{H}_{10}\text{O}_6$, when warmed, and the latter are easily reduced to hexoses $\text{C}_6\text{H}_{12}\text{O}_6$ by the use of sodium amalgam.³ In this way a pentose becomes a hexose. The reverse process of changing a hexose to a pentose takes place in like manner by way of the intermediate stage of the acid:

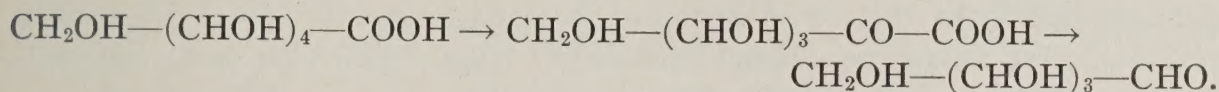


¹ Fischer, E. Untersuchungen über Kohlenhydrate und Fermente. Vol. 82. 1909.

² Kiliani. Ber. chem. Ges. 19: 767 and 1128. 1886; 20: 339. 1887.

³ Fischer, E. Ber. chem. Ges. 22: 2204. 1889; 23: 930. 1890.

When oxidized with hydrogen peroxide in the presence of ferric acetate¹ or mercuric oxide² or finally by electrolysis,³ carbonic acid is split off and a sugar of a lower series is formed:



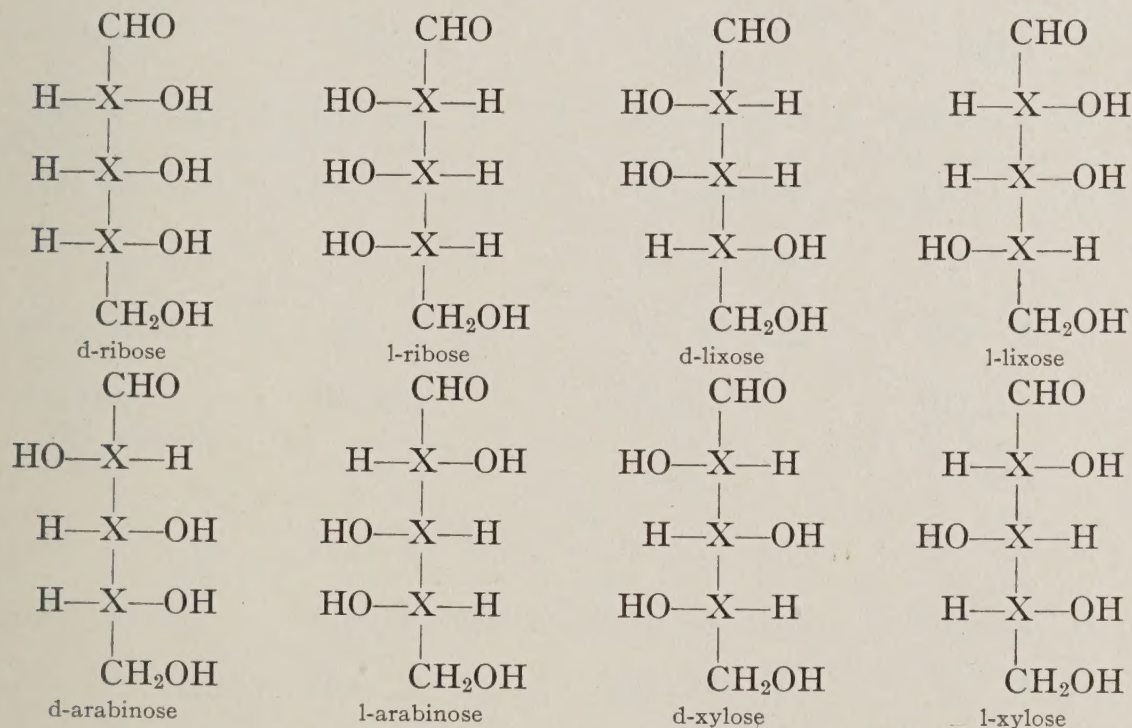
Glycolic aldehyde $\text{CH}_2\text{OH}-\text{CHO}$, the first member of the series, is considered to be an intermediate product of the synthesis of sugars in illuminated green leaves (*cf.* pp. 126-129). Syrup. Its para-nitrophenyl osazone (m.p. 311°) is especially characteristic.⁴

Trioses.—They are thought to be intermediate products of the formation of sugars from glycerine.⁵ **Glyceric aldehyde** (aldotriose) $\text{CH}_2\text{OH}-\text{CHOH}-\text{CHO}$ is only known in an optically inactive modification. Crystalline powder; m.p. 138°.

Dioxyacetone (ketotriose) $\text{CH}_2\text{OH}-\text{CO}-\text{CH}_2\text{OH}$; m.p. 68-75°. Unlike glyceric aldehyde, dioxyacetone readily yields an osazone with methyl phenyl hydrazine with m.p. 127-130°.⁶ The phenyl osazone of both these trioses crystallizes in prismatic flakes with m.p. 132° and decomposition point 170°.⁷

Tetroses are not found in plants.^j

Pentoses.—The variety of these sugars is considerable on account of stereoisomerism. There are 3 asymmetrical carbon atoms in aldopentoses and 8 isomeric compounds are possible:



Of these 8 aldopentoses, only two are found in plants—l-arabinose and l-xylose. Only traces can be detected in the free condition but they are com-

¹ Ruff. *Ibid.* 31: 1573. 1898; 32: 550. 1899.

² Guerbet. *Bull. Soc. Chem. de France* (4) 9: 431. 1908.

³ Neuberger, C. *Chem. Centralbl.* 1908 (I). p. 1165.

⁴ Wohl und Neuberger. *Ber. chem. Ges.* 33: 3107. 1900.

⁵ Kostytschew, S. *Zeitschr. f. physiol. Chem.* 111: 236. 1920. [It is also reported by Buston and Schryver (*Biochem. Jour.* 17: 470. 1923) that cabbage leaves contain a triose $\text{CH}_2\text{OH}-\text{CHOH}-\text{O}-\text{CH}_2\text{OH}$ which may be an intermediate product in photosynthesis.]

⁶ Neuberger, C. *Ber. chem. Ges.* 35: 964. 1902.

⁷ Fischer, E. und Tafel. *Ibid.* 20: 1089 and 3384. 1887; Piloty und Ruff. *Ibid.* 30: 1656. 1897.

^j However, see Spoeher. *Photosynthesis* for a description of the tetroses which have been obtained from glucosides.—Ed.

ponent parts of some carbohydrates of high molecular weight—the pentosans—which occur in cell walls (see below).

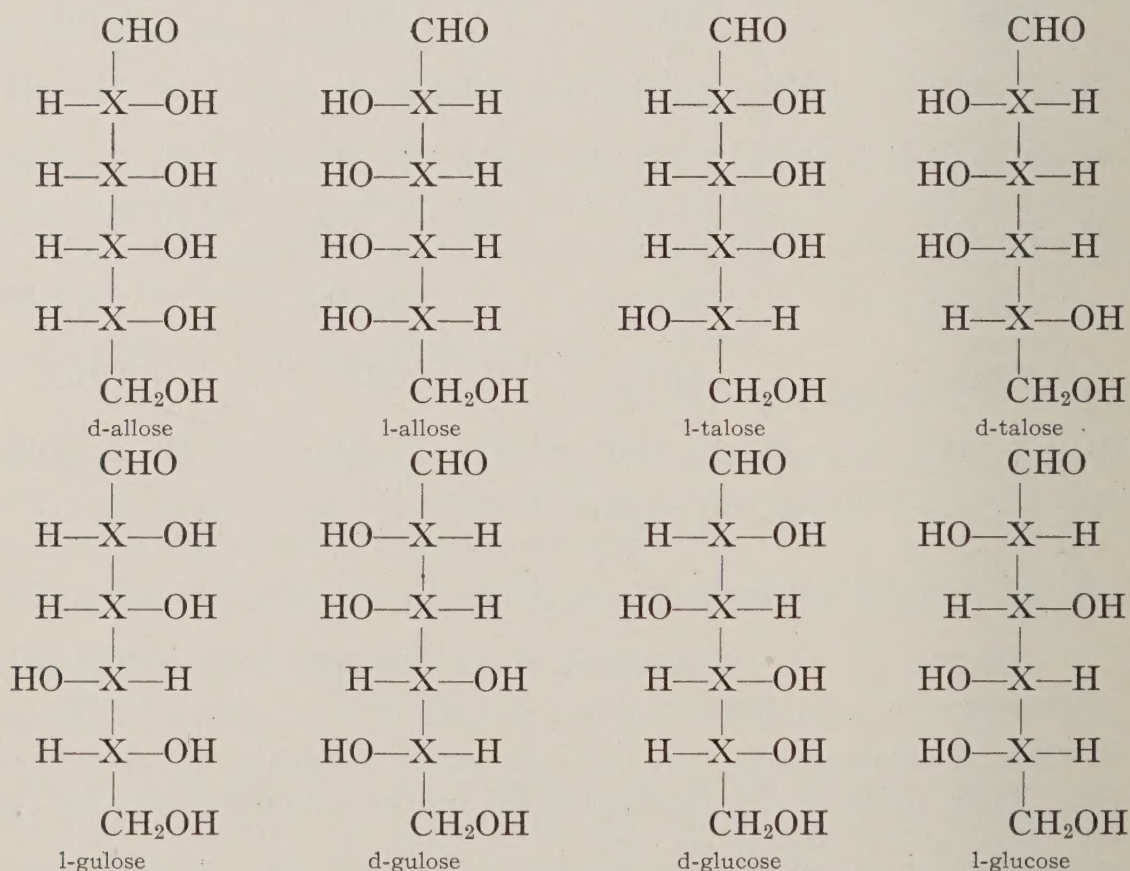
1-Arabinose is usually obtained from cherry gum.¹ It rotates polarized light to the right ($[\alpha]_D = +105$) but it gives l-gluconic acid in the HCN reaction. Its para-brom-phenyl-hydrazone² (m.p. 162°) and benzyl-phenyl-hydrazone³ (m.p. 170°) are especially characteristic.

1-Xylose is obtained by hydrolysis of wood, straw, etc. Very sweet prisms, $[\alpha]_D = +19^\circ$. Phenyl osazone, m.p. 161° . A characteristic derivative is the double salt of xylonic acid, $(C_3H_9O_6)_2 Cd + CdBr_2 + 2H_2O$. This is prepared by the oxidation of xylose with bromine water in the presence of cadmium carbonate.

In addition, plants contain methyl pentoses: **Fucose** from the cell membranes of *Fucus*, $[\alpha]_D = +76^\circ$; **Rhamnose** in many natural glucosides (phenyl osazone, m.p. 187°);⁴ **Apiose**, **rhodose**, **quinovose**—likewise in glucosides. They are the stereoisomers with the general formula $CH_3-(CHOH)_4-CHO$.

Hexoses are the most common and physiologically important sugars. Glucose (or fructose) is contained in almost all living plant cells.

The aldohexoses exist in the form of 16 stereoisomers:

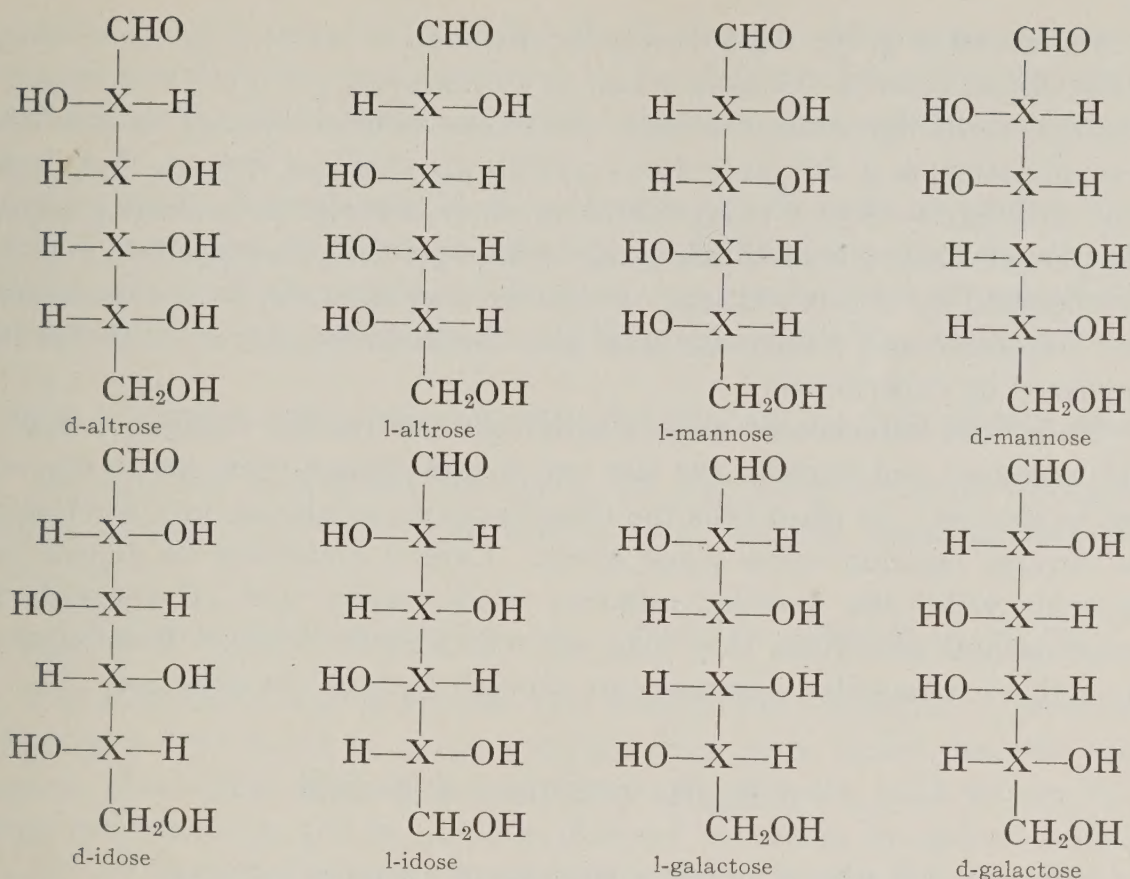


¹ Kiliani und Köhler. Ber. chem. Ges. 37: 1210, 1904.

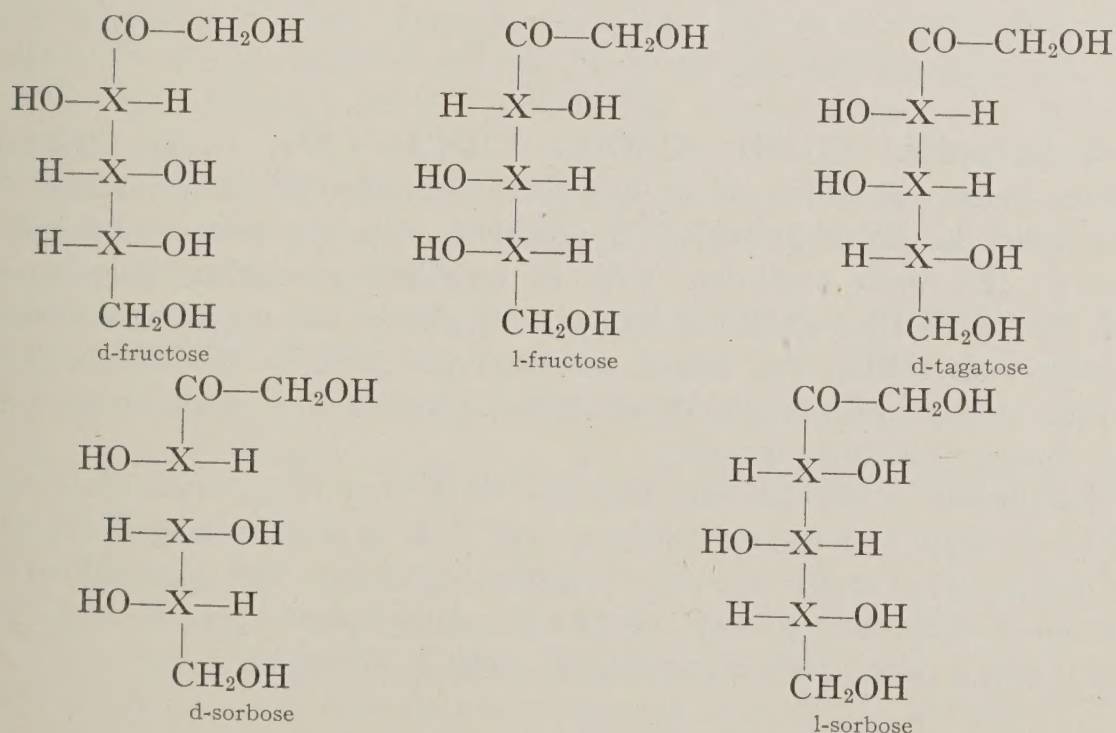
² Fischer, E. Ber. chem. Ges. 24: 4214. 1891; 27: 2490. 1894.

³ Lobry de Bruyn et van Eckenstein. Rec. trav. chim. des Pays-Bas. 15. 97 and 227. 1896.

⁴ Votocek. Ber. chem. Ges. 44: 820 and 823. 1911.



Of all these sugars, plants contain only d-glucose in the free condition. d-Mannose and d-galactose are also present as parts of polysaccharides, of cell walls and of glucosides. Like the pentoses, the ketohexoses could form 8 isomers but only 5 are known:

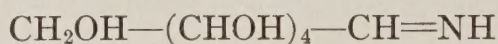


Only d-fructose occurs in plants in the free form. d-Sorbose is obtained by the bacterial oxidation of the alcohol sorbite which is stored in the berries of the mountain ash.¹

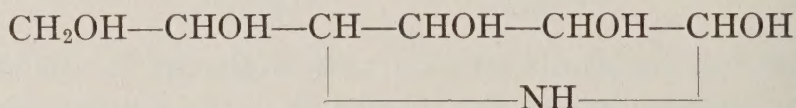
¹ Bertrand, G. Ann. chim. et physique (8) 3: 200. 1904.

d-Glucose or grape sugar is usually prepared in industry by the hydrolysis of starch (see below). Glucose occurs in almost every plant cell and frequently accumulates in high concentrations. With one molecule of water it crystallizes in small plates; m.p. 86° ; anhydrous crystals are obtained from methyl alcohol; m.p. 146° ; $[\alpha]_D = +52.5^{\circ}$; very soluble in water, less soluble in absolute alcohol. The phenyl osazone crystallizes in the form of golden-yellow needles; m.p. 205° accompanied by decomposition. It can be seen from the projection formulae that d-fructose and d-mannose must give the same osazone and this has been confirmed by experiments.

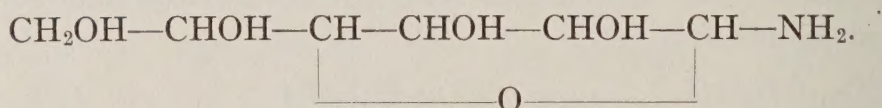
Under the influence of weak alkalis, glucose readily changes to fructose and mannose¹ and likewise the last two sugars change from one to the other and to glucose. In plant cells the transformation of glucose into fructose and the reverse reaction takes place easily. Careful treatment of glucose with ammonia yields the so-called osimines which readily split off ammonia and under natural conditions they may serve as transition stages from sugars to the amino compounds. Osimines are thought to have the structure



or



or



Glucosamine, $\text{CH}_2\text{OH}-(\text{CHOH})_3-\text{CHNH}_2-\text{CHO}$, is an interesting glucose derivative which occurs in considerable amounts in the cell walls of fungi² and in many proteins. Hydrochloric acid glucosamine has $[\alpha]_D = +72.5^{\circ}$. By weak oxidation (with Br in alkaline solution) gluconic acid $\text{CH}_2\text{OH}-(\text{CHOH})_4-\text{COOH}$ is formed from glucose and by stronger oxidation (boiling with HNO_3) the dibasic saccharic acid $\text{COOH}-(\text{CHOH})_4-\text{COOH}$. Further oxidation breaks the carbon chain. Reduction in a slightly acid solution changes glucose to d-sorbitol.

d-Fructose is likewise very common in the free form, crystallizes poorly and is strongly levo-rotatory, $[\alpha]_D = -92^{\circ}$. It is commercially prepared by the hydrolysis of inulin (see below), forms glucosazone with phenyl hydrazine but, more easily than glucose, it forms the methyl phenyl osazone (m.p. 158° – 160°)³ and it is not oxidized to gluconic acid by bromine.

¹ Lobry de Bruyn et van Eckenstein. Recueil des trav. chim. des Pays-Bas. 14: 203. 1895; 15: 92. 1896; 16: 257, 262, 274 and 282. 1897; 19: 9. 1900; and many others.

² Winterstein, E. Ber. chem. Ges. 27: 3113. 1894; 28: 167 and 1372. 1892; Idem. Zeitschr. f. physiol. Chem. 21: 134. 1895.

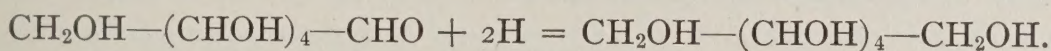
³ Neuberg, C. Ber. chem. Ges. 35: 959. 1902.

d-Mannose is prepared by hydrolysis of ivory nut (*Phytalephas*) fragments from the button industry. $[\alpha]_D = +14^\circ$. Its slightly soluble phenyl hydrazone (not osazone!), which forms directly in the cold, is especially characteristic; m.p. 204° .¹

d-Galactose is contained in the galactans of cell walls, in pectins and in various glucosides. It forms crystals more easily than the other simple sugars. $[\alpha]_D = +81^\circ$. Methyl phenyl hydrazone, m.p. 190° . It is characterized by the oxidation of galactose to mucic acid² $\text{COOH}-(\text{CHOH})_4-\text{COOH}$.

3. THE ALCOHOLS AND ACIDS WHICH CORRESPOND TO THE SIMPLE SUGARS

By reduction with sodium amalgam, the simple sugars change to the polyvalent alcohols of the same constitution. For example, mannite comes from mannose:



The polyvalent alcohols are also very often formed in plants. The alcohols which have been found in recent years are even more varied than the simple sugars. Polyvalent alcohols do not reduce copper oxide, yield neither hydrazones nor osazones, and are readily changed to sugars by gentle oxidation. They form characteristic acetals with aldehydes, especially with benzaldehyde. In many plants the polyvalent alcohols serve as reserve material which takes the place of sugar.

Glycerine $\text{CH}_2\text{OH}-\text{CHOH}-\text{CH}_2\text{OH}$, is formed by alcoholic fermentation and is a part of all fats and lecithins (Chap. VII). Very sweet syrup. Mixes with water and alcohol. Insoluble in ether. Forms tribenzoic ester $\text{C}_3\text{H}_5-(\text{O}-\text{CO}-\text{C}_6\text{H}_5)_3$ from benzoyl chloride in alkaline solution; m.p. 76° .

i-Erythrite $\text{CH}_2\text{OH}-(\text{CHOH})_2-\text{CH}_2\text{OH}$. Crystals, m.p. 112° . In lichens and a few algae.

Adonite, $\text{CH}_2\text{OH}-(\text{CHOH})_3-\text{CH}_2\text{OH}$, m.p. 102° . In *Adonis vernalis*.

The hexavalent alcohols $\text{CH}_2\text{OH}-(\text{CHOH})_4-\text{CH}_2\text{OH}$ are particularly common and include the following.

i-Dulcitate (m.p. 186°) is oxidized to galactose and then to mucic acid.

d-Mannite is common, especially in fungi; m.p. 166° ; $[\alpha]_D = +25^\circ$; is oxidized to fructose or mannose. Slightly soluble in cold alcohol. Tribenz-acetal³ $\text{C}_6\text{H}_8\left(\begin{smallmatrix} -\text{O} \\ -\text{O} \end{smallmatrix} \right) \text{CH}-\text{C}_6\text{H}_5)_3$, m.p. $207-208^\circ$, scarcely soluble in water.

d-Sorbite, needles, m.p. 110° , $[\alpha]_D = -1.73^\circ$.

d-Idite, m.p. 74° , $[\alpha]_D = -3.53^\circ$.

Among the septavalent alcohols found in plants are

d-Perseite, $\text{CH}_2\text{OH}-(\text{CHOH})_5-\text{CH}_2\text{OH}$, m.p. 188° ; and

Volemite, isomeric with the preceding, m.p. 155° , $[\alpha]_D = +2.65^\circ$.

¹ Storer. Bull. Bussey Inst. 3(II): 13. 1902.

² Fernan. Zeitschr. f. physiol. Chem. 60: 284. 1909.

³ Meunier. Compt. rend. 106: 1425 and 1732. 1888; 107: 910. 1888; Idem. Ann. chim. et physique (6) 22: 412. 1891.

It is interesting that the sugars which correspond to the septavalent alcohols have not been found in plants.^k

In addition to these aliphatic alcohols, there are also cyclic alcohols (see Chap. VII) which have not yet been transformed to sugars by chemical methods and which appear in living protoplasm by the oxidation of the foregoing alcohols with open chains.¹

Oxidation of the simple sugars gives the corresponding acids. Only the following acids have been found in plants up to the present time: **Gluconic acid**, $\text{CH}_2\text{OH}-(\text{CHOH})_4-\text{COOH}$, which is formed by the oxidation of sugar by the fungus *Aspergillus niger*;² also **glycuronic acid** and **galacturonic acid**. Glycuronic acid is a half-aldehyde of saccharic acid and galacturonic acid is a half-aldehyde of mucic acid. Their structure is expressed by the formula $\text{COOH}-(\text{CHOH})_4-\text{CHO}$. Glycuronic acid is found only in the form of glucoside derivatives—the glycuronides.³ A syrup which changes easily to the lactone, glycurone; m.p. 175° ; $[\alpha]_D = +18^\circ$. Galacturonic acid is a cleavage product of the pectins.⁴

4. DISACCHARIDES

Acetal-like compounds, consisting of two molecules of simple sugars less a molecule of water, are called disaccharides. In some of them the aldehyde properties are retained while in other both carbonyl groups are fixed. Disaccharides usually crystallize better than the simple sugars and can be purified more easily. They are split into their component parts by boiling with weak acids and by the action of plant enzymes. The synthesis of disaccharides from simple sugars seems to be an enzymatic process in plants.

Cane sugar or **saccharose**, $\text{C}_{12}\text{H}_{22}\text{O}_{11}$, produced by a great industry and important as a food, is very common in plants; its content is very high in the juice of the sugar beet, in sugar cane and in some other plants. Heating with dilute acids changes cane sugar to a mixture of equimolecular parts of glucose and fructose. The action is also brought about by the widespread enzyme, invertase, which gives with sugar a compound with acid properties.⁵ The hydrolyzed sugar is called invert sugar; it is levorotatory while cane sugar has $[\alpha]_D = +66.5^\circ$. The change of rotation of the solution is a quantitative measure of the process of inversion and has been very useful in studying the kinetics of enzyme reactions (Chap. I). Cane sugar does not reduce copper oxide, forms no osazone and has practically no aldehydic properties. For various reasons⁶ its formula is written:

^k A seven-carbon sugar called sedoheptose, has been reported by LaForge and Hudson. J. Biol. Chem. 30: 61. 1917; 42: 367 and 375. 1920. Another sugar that is related to perseite (=perseitol) is known as d-mannoketoheptose and was found by LaForge. Ibid. 28: 511. 1917.—Ed.

¹ On this point cf. Kumagawa, H. Biochem. Zeitschr. 131: 157. 1922.

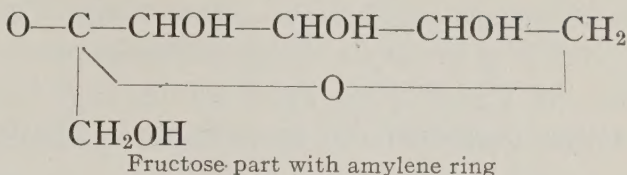
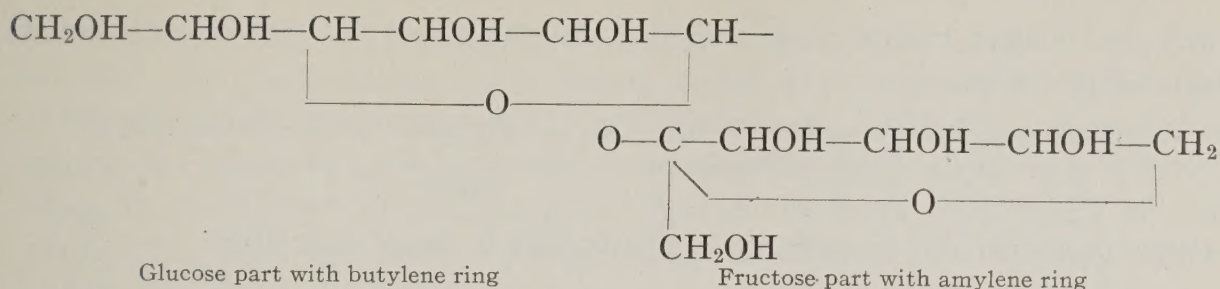
² Molliard, M. Compt. rend. 171: 881. 1922; 178: 41. 1924.

³ Goldschmidt und Zerner. Monatsh. f. Chem. 31: 439. 1910; Smolenski. Zeitschr. f. physiol. Chem. 71: 266. 1911.

⁴ Ehrlich, F. Chem. Zeit. 41: 197. 1917.

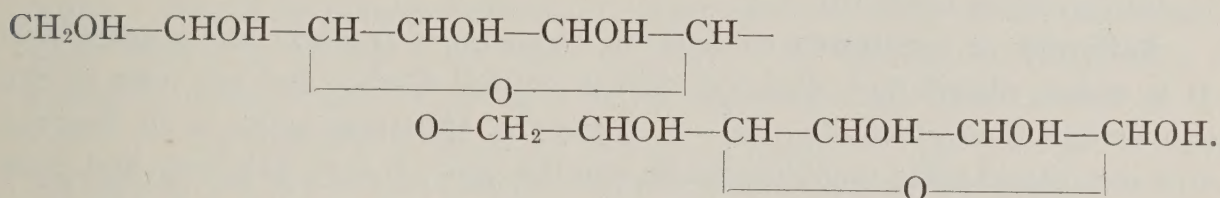
⁵ Michaelis, L. und M. Rothstein. Biochem. Zeitschr. 110: 217. 1920.

⁶ Haworth, W. and J. Mitchell. Jour. Chem. Soc. (London) 123: 301. 1923; Haworth, W. and W. Linnel. Ibid. 123: 294. 1923.



Although cane sugar is very soluble in water, it serves only as a reserve substance. Previous to inversion it does not take part in chemical reactions and, on account of its high molecular weight, it diffuses very slowly through the plasma membrane.

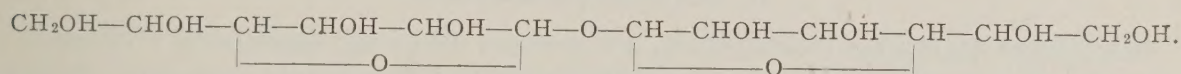
Maltose, $\text{C}_{12}\text{H}_{22}\text{O}_{11}$, consists of two molecules of glucose, so united that one aldehyde group is active. Maltose reduces copper oxide. Its phenyl osazone has m.p. 202° – 208° . It probably has the following structure:¹



$[\alpha]_D = +137^\circ$. Maltose is formed from starch by the action of the enzyme amylase and is split into two molecules of glucose by the enzyme maltase. Invertase does not work on maltose but dilute acids effect a complete hydrolysis.

Maltase was used for the first demonstration of reversed enzyme action.¹ Maltase in a concentrated syrup of glucose, in agreement with the law of mass action, effects a dehydration and forms the disaccharide revertose with $[\alpha]_D = +91.5^\circ$. A product similar to and perhaps identical with revertose² has been obtained from glucose by purely chemical devices. A method of purifying maltase preparations has recently been devised.³

Trehalose or **mycose**, $C_{12}H_{22}O_{11}$, likewise consists of two parts of glucose which are linked together as follows:⁴



$[\alpha]_D = +197$. It is particularly common in the fungi. It is not easily split by acids but trehalase, an enzyme which accompanies trehalose, readily changes it to glucose. Other hydrolytic enzymes do not attack trehalose.

Cellobiose or **cellose**, $C_{12}H_{22}O_{11}$, bears the same relation to cellulose that maltose does to starch. Cellobiose reduces copper oxide and forms a phenyl osazone with m.p. $208-210^{\circ}$, but it is not identical with maltose. $[\alpha]_D = +36.6^{\circ}$. In plants it is split only by the enzyme cellulase with the formation of

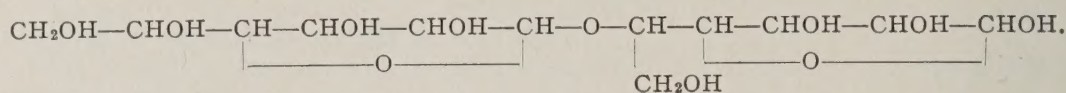
¹ Hill, C. Jour. Chem. Soc. (London) **73**: 634. 1898; *cf.*, however, page 42.

² **Armstrong.** Proc. Roy. Soc. London (B) **76**: 592. 1905.

³ Willstätter, R., F. Oppenheimer und W. Steibelt. Zeitschr. f. physiol. Chem. **110**: 232. 1920;
Willstätter, R. und W. Steibelt. *Ibid.* **111**: 157. 1920.

⁴ Haworth, W. and J. Mitchell. *loc. cit.*; Haworth, W. and W. Linnell. *loc. cit.*

glucose. Other hydrolyzing enzymes do not affect it. Its constitution is shown by the formula:

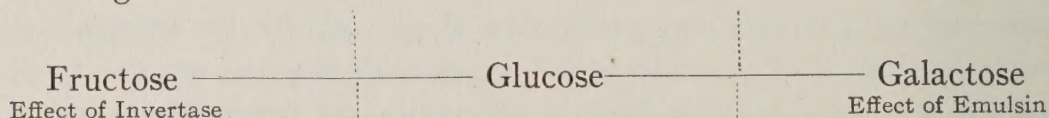


Other disaccharides are present in plants only in small quantities.

5. TRISACCHARIDES

These sugars have the formula $\text{C}_{18}\text{H}_{32}\text{O}_{16}$ and are formed of three molecules of simple sugars minus three molecules of water. Since there is no single form of union of the various sugars in the several trisaccharide molecules, the latter are, for the most part, completely hydrolyzed only in the presence of two different enzymes. Invertase usually splits off only fructose while emulsin removes cane sugar from the third component. Acids bring about complete hydrolysis when heated to 100° .

Raffinose or **melitriose** consists of d-glucose, d-fructose and d-galactose. It is widely distributed, dissolves well in methyl alcohol and has none of the properties of an aldehyde. $[\alpha]_D = +104.5^\circ$. Invertase splits it to fructose and the disaccharide melibiose while emulsin splits it into galactose and cane sugar. This shows that in the raffinose molecule the glucose is inserted between fructose and galactose:



Gentianose is decomposed into d-fructose and gentiobiose by the action of invertase. Gentiobiose consists of two molecules of glucose.

Melizitose has the same structure but a molecule of glucose comes off first. [According to Borland's American Medical Dictionary, melizitose comes from *Pinus larix* and yields fructose, glucose and galactose.]

Rhamninose has aldehydic properties and consists of two rhamnose units and one of galactose; its composition is $\text{C}_{18}\text{H}_{32}\text{O}_{14}$. $[\alpha]_D = -41^\circ$.

6. TETRASACCHARIDES

Stachyose,¹ $\text{C}_{24}\text{H}_{42}\text{O}_{21}$, crystallizes in small sweet flakes and consists of 1 molecule of d-fructose, 1 molecule of d-glucose and 2 molecules of d-galactose. $[\alpha]_D = +149^\circ$. Isomeric with it is **lupeose**² which has not yet been prepared in crystalline form.

7. POLYSACCHARIDES

The saccharides change properties as their molecular weights become larger. The disaccharides remind one of the simple sugars, while the tri- and tetra-saccharides are less sweet in taste, less soluble in water and more difficult to crystallize. Still larger complexes have not been prepared in crystalline form. In the plants we find polysaccharides with colloidal properties and high molecu-

¹ Schulze, E. und Planta. Ber. chem. Ges. 23: 1692. 1890; 24: 2705. 1891; Tanret, C. Bull. Soc. Chim. (III) 27: 948. 1902; Idem. Compt. rend. 136: 1569. 1903.

² Schulze, E. Ber. chem. Ges. 25: 2213. 1892; 43: 2230. 1910; Idem. Landwirt. Versuchs-Stationen. 41: 207. 1892; Idem. Chem. Zeit. 26: 7. 1902. Schulze considers that lupeose is a characteristic galactan (hemicellulose). Cf. also Schulze, E. und Godet. Zeitschr. f. physiol. Chem. 61: 294. 1909; Schulze, E. und Pfenninger. Ibid. 69: 367. 1910.

lar weights. They are practically insoluble in water, do not taste sweet, show no aldehydic properties but yield simple sugars when completely hydrolyzed. It often happens in chemistry that merely increasing the mass of the molecule results in a whole series of important *qualitative* changes in chemical properties. Study of the polysaccharides has shown that the methods of chemistry are not yet equal to work with amorphous substances. All that we know about starch and similar important substances is based on studies of the products of hydrolysis. The general empirical formula for polysaccharides is $(C_6H_{10}O_5)_n + H_2O$.^m This also covers all the lower saccharides. Thus the disaccharides $C_{12}H_{22}O_{11}$ can be expressed as $(C_6H_{10}O_5)_2 + H_2O$, trisaccharides $C_{18}H_{32}O_{16}$ as $(C_6H_{10}O_5)_3 + H_2O$, tetrasaccharides $C_{24}H_{42}O_{21}$ as $(C_6H_{10}O_5)_4 + H_2O$, etc.

Starch is the most important reserve carbohydrate of plants. It occurs in protoplasm in the form of stratified grains which are formed only in the plastids. The grains do not dissolve in cold water, swell in hot water, burst and form pastes. Starch becomes soluble after treatment with cold acids and through other analogous effects. The blue or violet color produced with iodine is especially characteristic of starch. Recent investigations have cleared up the matter of the chemical structure of starch. Maquenne¹ was the first to show that starch can be entirely changed to glucose without the formation of dextrans and other similar products. He distinguished between two polysaccharides in starch grains: the first is amylose which is soluble in water, gives a blue color with iodine and forms most of the grain; the second is amylopectin which does not dissolve in water, yields a violet color with iodine and forms the outer layer of the grain. The presence of swollen amylopectin in the solution of amylose gives the paste condition.

Hydrolysis (and probably the synthesis) of starch in plant cells is brought about by two enzymes. They are amylase (diastase) which splits starch to maltose, and maltase which splits maltose into two molecules of glucose. In the change of starch to sugar, the so-called **dextrans** are formed as intermediate or by-products. They are amorphous, dextrarotatory polysaccharides, readily soluble in water and not sweet to the taste.

Herzfeld and Klinger,² who have carried out new investigations of the nature of starch, believe that starch is only a certain condition of dispersion of dextrans—a conglomerate of molecules of these polysaccharides. For this reason the first starch-splitting enzyme—amylase—does not effect a chemical reaction but has only a physical action in the sense of an increased dispersion of the dextrans of which starch is formed. It will be explained later on that analogous conceptions are now appearing with respect to proteins and their cleavage by the enzyme pepsin. This "cleavage" is also considered to be a purely physical disruption and not a chemical process.

^m For a recent summary of our knowledge of the constitution of the polysaccharides see Irvine, J. C. Chem. Reviews. 1: 41-71. 1924.—Ed.

¹ Maquenne, L. Compt. rend. 138: 375. 1904; 146: 317 and 542. 1908; Idem. Ann. de chim. et de physique (8) 2: 109. 1904; Idem. Bull. Soc. Chim. de France (3) Vol. 35. 1906; Maquenne, Fernbach et Wolff. Compt. rend. 138: 49. 1904; Maquenne et Roux. Ann. de chim. et de physique. 9: 179. 1906; Roux. Compt. rend. 140: 440. 1905; 142: 95. 1906; Idem. Bull. Soc. Chim. de France (3) 33: 471, 723 and 788. 1905; Gruzewska. Compt. rend. 146: 540. 1908; 148: 578. 1909; 152: 785. 1911.

² Herzfeld, E. und R. Klinger. Biochem. Zeitschr. 107: 268. 1920; 112: 55. 1920; [cf. also Sideris. C. P. Plant Physiol. 3: 309-321. 1928].

More recent studies¹ lead to the conclusion that starch is put together from systems of rings which are said to be largely combinations of 14-membered rings which are two molecules of glucose joined by way of two atoms of oxygen. Moreover, Karrer assumes that the "starch molecule" contains at most only 6 glucoses and that starch does not have as high a molecular weight as it was once supposed to have. This hypothesis is not supported by experimental evidence. A recent view concerning the presence of phosphorus in the starch molecule² cannot be accepted because it is impossible to separate the starch grains from the parts of the plastids which contain phosphorus.

The mixture of enzymes which changes starch to glucose is called "diastase." It was the first enzyme to be discovered in organisms.³ In spite of the large number of investigations which have been devoted to diastase (Chap. I), its chemical aspects have not been made clear.

For a long time starch was regarded as a carbohydrate peculiar to plants containing chlorophyll but recently it has been found that starch-like substances are produced by molds.⁴

Inulin replaces starch in some *Compositae*. It is an interesting fact that inulin is often formed in the root at the expense of dextrarotatory sugar entering from the stem,⁵ although inulin itself is levorotatory. $[\alpha]_D = -39^\circ$. It is not colored by iodine and yields only d-fructose when hydrolyzed. Diastase does not attack inulin which is hydrolyzed only by inulase, a specific enzyme. The first products are **levulins** *i.e.*, substances analogous to dextrans except that they are levorotatory. Inulin is soluble in hot water. Its molecular weight is about 9 times that of fructose.⁶ According to Irvine,⁷ inulin consists of γ -fructose (p. 233). There are a few less widely distributed polysaccharides which yield only d-fructose when hydrolyzed.

Glycogen replaces starch in animals because it also functions as a reserve carbohydrate.⁸ Glycogen is found in fungi but this glycogen does not seem to be identical with animal glycogen.⁹ Amorphous powder, easily dissolved in water. $[\alpha]_D = +199^\circ$. The molecular weight of glycogen is said to be high.¹⁰ It yields a brown color with iodine. Only d-glucose is formed by hydrolysis.

According to the latest information,¹¹ glycogen is only a dispersed form of colloidal starch. The difference between starch and glycogen is not in its chemical structure but in the colloidal condition of the two carbohydrates. In yeast, glycogen often forms a third of its dry weight. In living cells, the hydrolysis of glycogen is brought about by the enzyme glycogenase.

¹ Pringsheim, H. und A. Langhaus. Ber. chem. Ges. 45: 2533. 1912; Pringsheim, H. und K. Goldstein. *Ibid.* 55: 1446. 1922. Cf. especially Karrer, P. Ergeb. d. Physiol. 20: 433. 1922.

² Thomas. Biochem. Bull. 3: 403. 1914; cf. also Kerb, J. Biochem. Zeitschr. 100: 3. 1919.

³ Payen et Persoz. Ann. de chim. et de physique (2) 53: 73. 1833.

⁴ Boas, F. Biochem. Zeitschr. 78: 308. 1917; 86: 110. 1918; *Idem.* Ber. bot. Ges. 37: 50. 1919; [Chrzaszcz, T. und D. Tiukow. Biochem. Zeitschr. 207: 39-52. 1929].

⁵ Colin. Rev. gén. bot. 31: 75. 1919. Like starch, inulin is probably not a single substance: cf. Dean. Amer. Chem. Jour. 32: 69. 1904.

⁶ Pringsheim, H. und A. Aronowsky. Ber. chem. Ges. 55: 414. 1922.

⁷ Irvine, J. C. Jour. Chem. Soc. (London) 123: 518 and 898. 1923.

⁸ Bernard, Cl. Compt. rend. 41: 461. 1855.

⁹ Errera. Bull. de l'acad. de Bruxelles (3) Vol. 4. 1882; *Idem.* Compt. rend. 101: 253. 1885; Salkowski, E. Ber. chem. Ges. 27: 3325. 1894; *Idem.* Zeitschr. f. physiol. Chem. 92: 75. 1914.

¹⁰ Gruzewska. Compt. rend. 138: 1631. 1904.

¹¹ Herzfeld and Klinger. *loc. cit.*; Karrer. *loc. cit.*; Irvine. *loc. cit.*

Cellulose is the principal substance in the cell walls of plants containing chlorophyll and in absolute amounts is undoubtedly superior to all other organic substances on the surface of the earth. Pure cellulose is a latent-crystalline substance which dissolves in no solvent except Schweizer's reagent¹ which is an ammoniacal solution of cupric oxide. Dilute acids do not affect cellulose but concentrated sulphuric acid at 100–120° changes it all to d-glucose² (to prevent losses it is necessary to let the acid work first in the cold and then to heat it in the autoclave). The disaccharide cellobiose (see above) appears as an intermediate product of the hydrolysis of cellulose. According to Karrer (*loc. cit.*) cellulose, like starch, inulin and glycogen, is a system of cyclic compounds which are composed of sugar anhydrides. It also appears that the cellulose molecule includes maltose unions as well as cellobiose.ⁿ

In some plants, especially in parasites, there is the enzyme cytase which hydrolyzes cellulose.

Only the cell walls of young plants contain pure cellulose. Age brings on the appearance of their esters and the products of their methylation and oxidation. On account of its availability, light weight and firmness, cellulose is the basis of various technical industries.

Lignin and **lignocellulose** are substances in woody cell walls; their constitution has not been determined. Lignin is a product of the methylation and esterification of cellulose; it contains aromatic rings and acetyls (framework of acetic acid $\text{CH}_3\text{-CO}$). It is generally supposed that lignin forms ether-like unions with cellulose.³ These ethers are called lignocelluloses.

Suberin, the substance of corky cell walls, is not closely connected with the carbohydrates. It is insoluble in concentrated sulphuric acid but dissolves in alcoholic lye.⁴ Suberin seems to be a collective term. The corky substance is composed of different alcohols and acids. The composition of cutinized cell walls is no less diverse.

8. HEMICELLULOSES

It was once thought that unligified or non-corky cell walls are built of pure cellulose but the outstanding investigations of E. Schulze⁵ disproved this view. It appears from his papers that in cell walls there are various polysaccharides which are hydrolyzed by dilute sulphuric acid and are composed of mannose, galactose, arabinose and xylose. These substances are often reserve carbohydrates. Now the theory of the cell wall must be reformed inasmuch as it

¹ Schweizer, E. Jour. prakt. Chem. 76: 109 and 344. 1857.

² Ost und Brodtkorb. Chem. Zeit. 35: 1125. 1911; Ernest. Ber. chem. Ges. 39: 1947. 1906; Irvine, J. C. and Soutur. Jour. Chem. Soc. (London) 117: 1489. 1920; Irvine and Hirst. *Ibid.* 121: 1585. 1922; Monier-Williams. *Ibid.* 119: 803. 1921.

ⁿ During recent years the molecular structure of cellulose as it occurs in the cell walls of plant fibers has been studied by means of x-ray diffraction patterns. A summary of these investigations is given by Sponsler (Amer. Jour. Bot. 15: 525–536. 1928) who has found that such cell walls consist of a lattice work of $\text{C}_6\text{H}_{10}\text{O}_5$ units arranged in parallel chains that are evenly spaced.—*Ed.*

³ Cross and Bevan. Jour. Chem. Soc. (London) 41: 694. 1883; Idem. Phar. Jour. Trans. 3: 570 1884; Idem. Cellulose: an outline of the structural elements of plants. 1903.

⁴ Gilson. La Cellule. 6: 87. 1890.

⁵ Schulze, E. Ber. chem. Ges. 22: 1192. 1889; 23: 2573. 1890; 24: 2277. 1891; Idem. Chem. Zeit. 19: 1465. 1895; Idem. Landwirt. Jahrb. 21: 72. 1892; 23: 1. 1894; Idem. Zeitschr. f. physiol. Chem. 16: 387. 1892; 19: 38. 1894; 61: 307. 1909; and many others.

has become known that the substances of the cell walls are not exclusively structural materials but are also reserve materials⁰ under certain conditions. On the basis of recent data,¹ most of the carbon compounds assimilated in the light are often deposited in the cell walls and not changed to starch. Hemicelluloses are abundant in seeds but are also generally distributed in both lower and higher plants. The cell membrane of yeast is largely composed of hemicelluloses.² All hemicelluloses are soluble in glycerine at 300.⁰ In the physiological relations, the mannose in them plays the rôle of a reserve sugar.

Mannans are hemicelluloses which are found in the palms. The bony Brazil nut (*Phytelephas*) is nothing but a reserve carbohydrate, most of which is easily hydrolyzed by powerful enzymes of the seed with the formation of one part d-fructose to 20 parts of d-mannose³; however, part of the seed mannans are split with difficulty. In other cases hydrolysis of hemicelluloses yields d-mannose and d-galactose (mannogalactans).

Galactans are hemicelluloses which yield chiefly galactose when hydrolyzed. According to Schulze, they are particularly common in the *Leguminosae*.

Pentosans are hemicelluloses which are composed chiefly of pentoses. They are divided into the **xylans** and **arabans**. The former are common in wood, bran and straw and yield l-xylose upon hydrolysis; the latter yield l-arabinose. The pentosans are formed indirectly from polysaccharides containing hexoses.⁴ Cross, Bevan and Smith⁵ believe that the aldehyde group of hexose thereby remains fixed and intact, and that the primary alcohol group at the opposite end of the carbon chain is oxidized, decarboxylized and escapes as CO₂. Therefore l-xylose must come from d-glucose and l-arabinose from d-galactose (*cf.* the projection formulae on pp. 235 to 237).

Amyloid is not identical with the hemicelluloses, according to Schulze.⁶ Like starch, it stains blue with iodine. It swells in cold water and by hydrolysis yields various sugars, largely d-galactose and l-xylose. It is found in many seeds.

Lichenin occurs in lichens and likewise swells easily in water but on hydrolysis it yields only d-glucose besides cellobiose as an intermediate product.⁷ In the cell walls there are also methyl pentosans and other polysaccharides which are interesting materials for chemical investigations.

Chitin, a polysaccharide containing nitrogen, occurs in the cell walls of the higher fungi. Yields glucosamine on hydrolysis. Insoluble in alkalies and soluble only in concentrated sulphuric and hydrochloric acids with the formation of a brown color. $[\alpha]_D$ in conc. sulphuric acid = +56°. Nitrogen content about 6%.⁸

⁰ The recent paper of **Murneck** (Plant Physiology. 4: 251-264. 1929) gives support to this idea and a discussion of the chemistry of the hemicelluloses.—*Ed.*

¹ **Körösy**. Zeitschr. f. physiol. Chem. 86: 368. 1913.

² **Meigen und Spreng**. Zeitschr. f. physiol. Chem. 55: 62. 1908; **Spreng**. Diss. Freiburg. 1906.

³ **Iwanow, S. L.** Jour. f. Landwirt. 56: 217. 1908.

⁴ **Cross and Smith**. Chem. News. 74: 177. 1896.

⁵ **Cross, Bevan and Smith**. Ber. chem. Ges. 28: 1940. 1895.

⁶ **Schulze, E.** Zeitschr. f. physiol. Chem. 19: 38. 1894.

⁷ **Pringsheim, H. und J. Leibowitz**. Zeitschr. f. physiol. Chem. 131: 262. 1923.

⁸ **Scholl, E.** Monatsschr. f. Chem. 29: 1023. 1908.

9. PECTINS

Unlike the hemicelluloses, these have a jelly-like or slimy consistency. Their chemical composition has been determined only in recent years. The framework of pectins is tetragalacturonic acid, a galacturone (analogous to the glycurones mentioned above), which decomposes into four molecules of galacturonic acid (half-aldehyde of mucic acid) $\text{COOH}-(\text{CHOH})_4-\text{CHO}$. Tetragalacturonic acid occurs in pectins in union with araban and d-galactose.¹ According to Ehrlich's results, galacturonic acid is very common in plants since it occurs in all pectins. The pectins are split by the specific enzyme pectinase.² They are the principal substance in the middle lamellae of cell walls.³ Hence hydrolysis of the pectin substance is accompanied by a maceration of the tissue. The process of steeping flax is based on maceration by bacteria.^p

10. GUMS AND MUCILAGES

These are secretion products of diseased or injured tissues.⁴ The gums are considered to be water-soluble and the mucilages swell in water. On hydrolysis the gums yield l-arabinose, d-galactose and acids of undetermined structure.⁵ The high content of d-galactose in some gums is shown by the output of mucic acid when they are oxidized with nitric acid.⁶ In the slimy secretions there are many remnants of destroyed cell walls, enzymes and other substances. It would be most interesting to make a detailed biochemical study of the gums and mucilages which are secreted from injured plants. Owing to the consistency of the medium, it can be supposed from the difference between the carbohydrates of the sap and those of the cell wall and from the presence of enzymes in the sap, that powerful enzymatic processes are going on here and presumably they may also be of a synthetic character.

It can hardly be doubted that the flow of gums and mucilages is induced not by bacterial but by traumatic effects.⁷ Various substances exert either a stimulating or a depressing effect on this process.⁸

Gum arabic, a product secreted by some species of *Acacias*, is not salted out and yields l-arabinose and d-galactose on hydrolysis.

¹ Ehrlich, F. *Chem. Zeit.* 41: 197. 1917; [Ehrlich, F. und A. Kosmahly. *Biochem. Zeitschr.* 212: 162-239. 1929.]

² Bourquelot, E. et Hérissé. *Compt. rend.* 127: 1911. 1898; *Idem.* *Jour. phar. et chim.* (6) Vol. 9. 1899. [According to work reported by Willaman, J. J. *Univ. Minn. Stud. Biol. Sciences.* 6: 333-341. 1927, plants contain three enzymes which attack pectins. *Pectosinase*, later called propectinase, dissolves the middle lamella of plant tissues and is very important in parasitism. *Pectase* coagulates soluble pectins to a gel in the presence of a calcium (or barium or strontium) salt. *Pectinase* hydrolyzes soluble pectins and gels to reducing sugars.—*Ed.*]

³ Payen. *Compt. rend.* 43: 769. 1856.

^p For the decomposition of pectin by bacteria, see Coles (*Plant Physiology.* 1: 379-385. 1926) and Norman (*Ann. Bot.* 43: 233-243. 1929).—*Ed.*

⁴ Lutz. *Compt. rend.* 150: 1184. 1910; cf. also Jodin et Boucher. *Ibid.* 146: 647. 1908; Frank. *Ber. bot. Ges.* 2: 321. 1884; Savastano. *Compt. rend.* 99: 987. 1884.

⁵ O'Sullivan, C. *Jour. Chem. Soc. (London)* 45: 41. 1884; 59: 1029. 1891; *Idem.* *Proc. Chem. Soc.* 17: 156. 1901; *Idem.* *Chem. News.* 64: 271. 1891.

⁶ Maumené. *Bull. Soc. Chim. de France* (3) 9: 138. 1893; Kiliani. *Ber. chem. Ges.* 19: 3030. 1886. The chemical nature of the gums and mucilages is probably similar to that of the pectins.

⁷ Beijerinck und Raut. *Centralbl. f. Bakt. (II)* 15: 366. 1905; Rathay. *Ibid.* 2: 620. 1896; Mangin. *Compt. rend.* 119: 514. 1895.

⁸ Ruhland, W. *Ber. bot. Ges.* 24: 393. 1906; 25: 302. 1907; Sorauer, P. *Landwirt. Jahrb.* 39: 259. 1910; 41: 131. 1911; 42: 719. 1912.

Gum tragacanth, a valuable product of *Astragalus*, is only partly soluble in water, contains 37% pentoses, and yields arabinose, xylose and fructose on hydrolysis.

Cherry gum is only partly soluble in water and contains 50% arabinose.

II. GLUCOSIDES

It was pointed out above that polysaccharides are acetal-like compounds of the carbonyl group of a simple sugar with hydroxyl groups of another sugar. This same union can also be made with different alcohols not of a sugary nature and the products of these reactions are called glucosides. The similarity between the natural glucosides and the di-, but especially the trisaccharides becomes evident when studied by the action of hydrolyzing enzymes.

Emulsin, a very common plant enzyme, splits both natural glucosides and trisaccharides (p. 242). This indicates a like union between the separate parts of these substances. On the contrary, the saccharides formed of simple sugars in unlike manner, such as cane sugar and maltose, are not split by the same enzyme. In general we distinguish between α -glucosides, which are dextrarotatory and split by the enzymes of yeast, and β -glucosides which are levorotatory and split only by emulsin.¹ The latter does not affect α -glucosides and appears to consist of three separate enzymes.² It would be more exact to use the name β -glucosidase for the plant enzyme which hydrolyzes β -glucosides. The pioneer syntheses of glucosides by enzymes, which were carried out by Bourquelot and his co-workers³ and which provide the most important evidence for the reversibility of enzyme action, have been considered in Chap. I.

The natural glucosides are numerous and widely distributed in plants. The physiological significance of glucosides appears to be that they serve as reserve material not only for the sugars but also for the various components to which they are bound. Very often these components are strong poisons which do not affect the protoplasm as long as they are held in the form of glucosides. However, the glucosides themselves often have poisonous properties and many of them are used in medicine. In addition unstable, easily oxidized substances are often stored as reserve material in the form of glucosides and are thereby protected from oxidation.

In the natural glucosides there is usually an aromatic nucleus which either remains independent or forms part of the structure of complicated rings such as the indol or flavone rings and the like. Some glucosides have a complicated structure and a high molecular weight so that not all members of this class of compounds in plants, and not even the cleavage products into which they break

¹ Fischer, E. Ber. chem. Ges. 27: 2985. 1894; 28: 1145. 1895; Idem. Zeitschr. f. physiol. Chem. 26: 60. 1898. It is assumed that there are two modifications of glucose which differ in the angle of rotation of polarized light; α -glucose has a strong effect while β -glucose is weaker in its action. The first is a part of α -glucosides and the second of β -glucosides. On this point see Armstrong, S. F. Jour. Chem. Soc. (London) 83: 1305. 1904; Hudson and Paine. Jour. Amer. Chem. Soc. 31: 124 2. 1909.

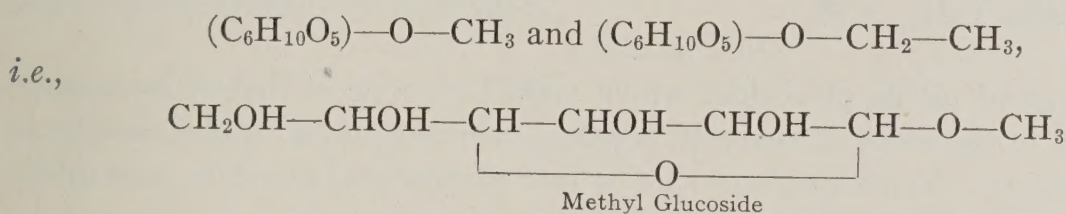
² Armstrong and Horton. Proc. Roy. Soc. London. 80: 321. 1908; Caldwell and Courtauld. Ibid. 79: 350. 1907.

³ Bourquelot, E., Hérissé et M. Bridel. Compt. rend. 156: 491. 1913; Bourquelot et Verdon. Ibid. 154: 957 and 1264. 1911; Bourquelot et Bridel. Ibid. 155: 437, 523 and 854. 1912; and others; cf. p. 42.

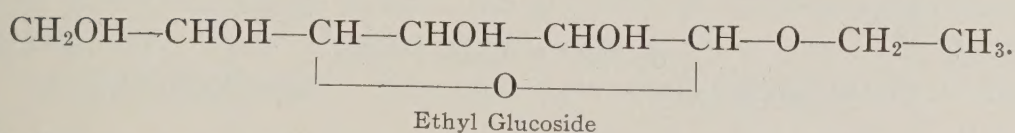
up when hydrolyzed, have been determined. If we summarize all the present knowledge we reach the conclusion that the formation of glucosides seems to be one of the most important physiological functions of sugars in plants.

Only a few especially characteristic and common glucosides are described here.¹

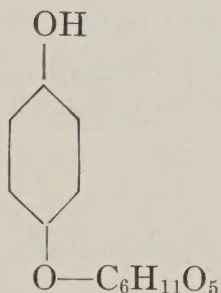
β -Methyl glucoside, α -ethyl glucoside and β -ethyl glucoside are examples of the synthetic glucosides which were prepared by Bourquelot and his co-workers with the aid of plant enzymes. Under these conditions a yield of 81% was obtained for the first glucoside. They are all crystalline, optically active substances which are related to sugar and are hydrolyzed by the suitable enzymes of yeast and almonds. Their taste is momentarily sweet, then bitter. Their structure is:



and

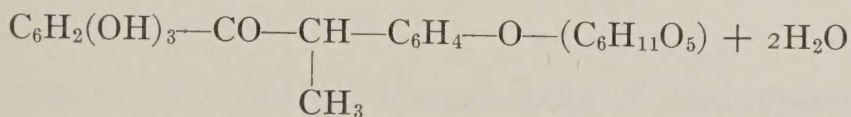


Arbutin



occurs in many plants and can serve as a representative of the simple plant glucosides which contain an aromatic nucleus in addition to sugar. Arbutin is composed of one glucose and hydroquinone (p-diphenol)² which appear on hydrolysis. M.p. 188°.

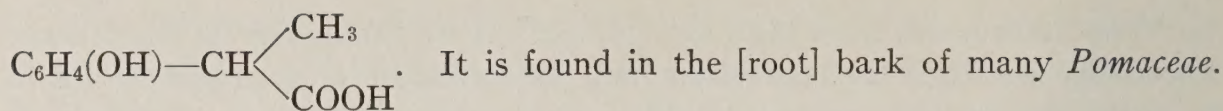
Phloridzin



contains two aromatic rings and decomposes into glucose and phloretin. The latter hydrolyzes to phloroglucin $C_6H_3(OH)_3$ and p-hydroxyhydratropic acid

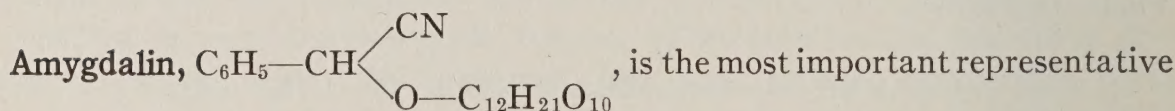
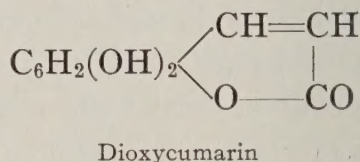
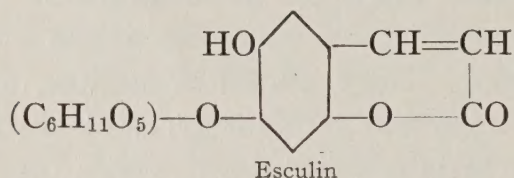
¹ Complete data about the glucosides can be found in the book by **van Rijns** entitled *Die Glucoside* (1900). Later facts appear in **Abderhalden's** *Biochem. Handlexikon*, Bd. 2 (1911) and Bd. 8 (1914) [and in **Armstrong** "The Carbohydrates and the Glucosides" now in the 4th edition.]

² **Strecker**. *Liebigs Ann. d. Chem.* 107: 228. 1858.

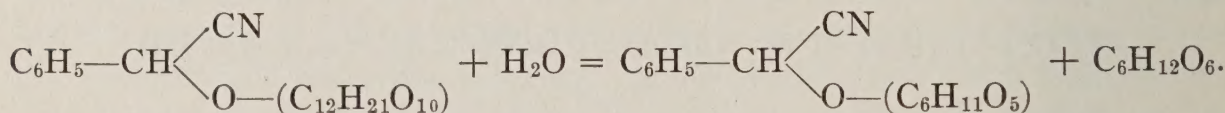


It disappears in water at 108° ; m.p. 170° .

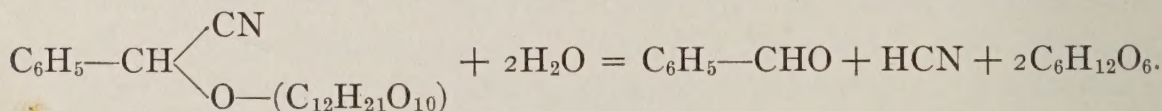
Esculin. From the bark of the horse chestnut and other plants. Sublimes easily. Enzymes split it into glucose and esculetin (dioxycumarin):



of the group of nitrile glucosides which yield hydrocyanic acid on hydrolysis. It is found in the seeds of almonds, peaches, cherries, plums, apples, mountain ash and others. Yeast enzymes split it into glucose and mandelic acid nitrile glucoside.

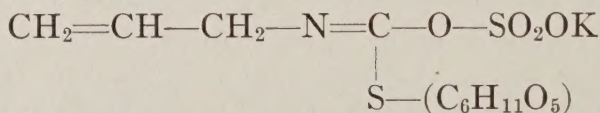


Thus these enzymes can break the bond between two glucose molecules in the amygdalin molecule and remove one of them but they are unable to separate the aromatic nucleus from sugar. Emulsin, the enzyme of bitter and sweet almonds, splits amygdalin into two glucose molecules, benzaldehyde and hydrocyanic acid:

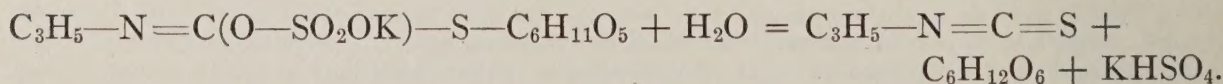


The nitrile glucosides seem to be important in plants as the storehouse of hydrocyanic acid which is split off as needed and used in various synthetic processes *e.g.*, to increase the length of carbon chains (p. 234).

Sinigrin



is a typical thioglucoside in which sugar is held not by an oxygen link but by sulphur. It is levorotatory and the m.p. is 126° . The enzyme myrosin¹ decomposes it into d-glucose, isothiocyanate ester of allyl alcohol, and potassium bisulphate:

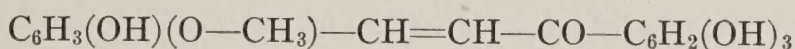


¹ Bussy. Ann. de chim. et de pharmacie. 34: 223. 1840.

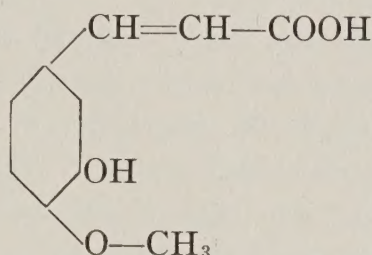
In the *Cruciferae* are found a few analogous glucosides in which the sugar is bound through sulphur.

Some glucosides have high molecular weights and their structure is still undetermined. These include the widely distributed:¹

Hesperidin $C_{50}H_{60}O_{27}$ (mol. wt. 1092); crystalline; soluble in water, ether and benzene with difficulty; readily soluble in alcohol; m.p. 251° . Decomposes into glucose, rhamnose and hesperitin,² a phloroglucin derivative (not an ester!) of hesperitinic acid:



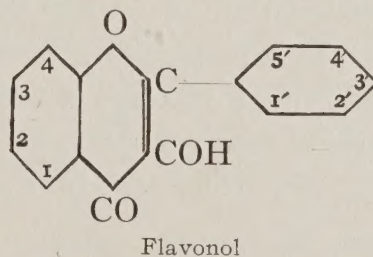
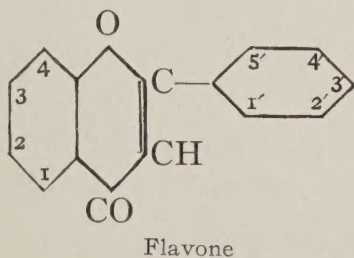
Hesperitinic acid is a methyl ester of caffeic acid



Saponins³ are numerous, amorphous, poisonous glucosides which are easily dissolved in water and which have the property of forming soapy-opalescent, frothy solutions. Their structural formulae have not yet been determined. They contain no nitrogen. Saponins are widely distributed in the plant world.

The sugars in saponins are glucose, galactose, arabinose and methyl pentoses. When the sugar is removed the residue is an insoluble substance—sapogenin. This is often a glucoside. The saponins yield insoluble precipitates when treated with lead acetate and barium hydroxide. Some of them can be salted out of solution. The probable general formula for saponins is $C_nH_{2n-8}O_{10}$.⁴

Flavone glucosides, together with the carotin derivatives (p. 83) and the still uninvestigated anthochlors [anthoxanthins], make up all the yellow and orange-yellow pigments of plants. The derivatives of the flavones and the flavonols (oxyflavones) are commercially important dyestuffs.



The formula for flavone was determined by the synthesis from benzoic acid and ortho-acetophenone.⁵

¹ Borodin. Arb. St. Petersburger Naturforscherges. 1883 (Russian).

² Tanret. Bull. Soc. Chem. de France. 49: 20. 1888.

³ Bussy. Jour. de pharmacie. 19: 1. 1833. [For a summary of the chemical, physical and biological properties of the saponins see Kofler. Protoplasma. 7: 106-128. 1929.]

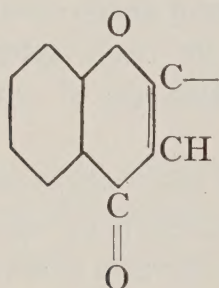
⁴ Kobert. Arch. f. exp. Path. und Pharmakol. 23: 233. 1887.

⁵ v. Kostanecki und Feuerstein. Ber. chem. Ges. 31: 1757. 1898; v. Kostanecki und Tambor. Ibid. 33: 330. 1900.

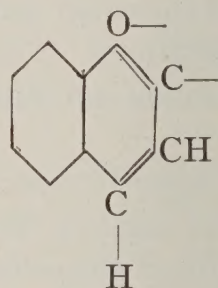
The principal component of the flavone glucosides is **quercetin**, 1, 3, 3', 4'-tetraoxyflavonol.¹ For example, in the bark of many plants is found the glucoside **rutin**, of lemon-yellow color, which is very easily dissolved in cold water like all flavone derivatives.² On hydrolysis it decomposes into quercetin and rhamnose. Another glucoside, **xanthorhamnin**, is hydrolyzed to galactose, rhamnose and rhamnetin, the trimethyl ether of quercetin.³ Many other analogous substances have been described by Perkin⁴ and his co-workers. The flavonol and flavone derivatives occur in plants not only as glucosides but also in the free condition, such as **chrysin** 1, 3-dioxyflavone⁵ and **luteolin** 1, 3, 3', 4'-tetraoxyflavone.⁶ All are insoluble in water and ether, soluble in hot alcohols and alkalies, easily crystallized and are yellow in color.

Anthocyans are glucosides which contain oxonium bases of derivatives of the pyrilium ring. They are important in plants as pigments of flowers, variegated leaves and fruits.

The nature of anthocyan was made clear by the work of Willstätter and his co-workers after various authors had reported faulty experiments. These papers could serve as standards for the isolation of new, unknown, natural products and the determination of their nature. The anthocyan so far studied prove to be chains of sugars, especially glucose, galactose and rhamnose, with peculiar pigments, the anthocyanidins, which are related to the flavone derivatives but have a different structure. In place of the chromon portion



they contain the double ring



i.e., they lack the carbonyl group CO. However, they contain the quinoid oxonium group⁷ which has basic properties because the tetravalent oxygen can join with acids through its free valence. On the other hand, the phenol groups in the anthocyanidins have acid properties. Thus the anthocyan are amphotytes. Their compounds with bases (with potassium in the corn flower under natural conditions) are blue while the acid compounds are bright red. The free bases form an intramolecular salt of a violet color but it is rapidly decolorized by isomerization and change of the oxygen from tetravalent to divalent (this bleaching was formerly held to be a reduction of the anthocyan). Outside of the plant, the transformation of flavone derivatives into anthocyanidins

¹ v. Kostanecki. Ber. chem. Ges. 26: 2901. 1893; Kostanecki, Lampe und Tambor. Ibid. 37: 1402. 1904.

² Schmidt, E., Waliaschko, Branns und Wunderlich. Arch. f. Pharm. 242: 210, 225, 383, 547 and 556. 1904; 246: 224, 241 and 256. 1908.

³ Herzig, J. Monatsh. f. Chem. 6: 889. 1885; 9: 548. 1888; 10: 561. 1889.

⁴ Perkin, A. G. Jour. Chem. Soc. (London) 71: 1131. 1897; 95: 2181. 1909; Perkin and Martin. Ibid. 71: 818. 1897; Perkin and Pilgrim. Ibid. 73: 267. 1898; Perkin and Hummel. Ibid. 77: 424. 1900; Perkin. Ibid. 81: 205. 1902; and others.

⁵ v. Kostanecki. Ber. chem. Ges. 26: 2901. 1893; 32: 2448. 1899; 37: 3167. 1904.

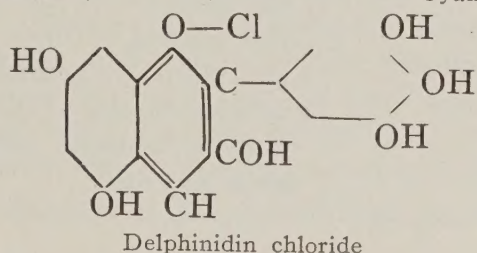
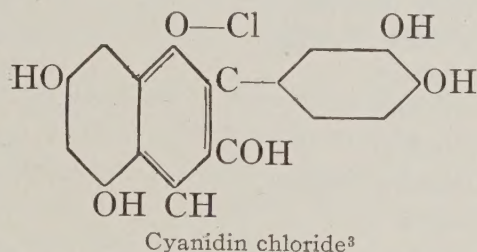
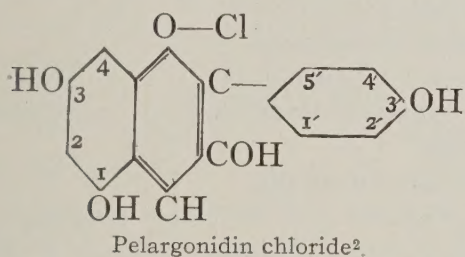
⁶ Perkin, A. G. Jour. Chem. Soc. (London) 69: 206. 1896; Idem. Proc. Chem. Soc. 15: 242. 1900; 16: 181. 1900.

⁷ Perkin, W. H., R. Robinson and M. R. Turner. Jour. Chem. Soc. (London) 93: 1085. 1908:

through direct reduction of the carbonyl group of the pyrone ring has been carried out only as a side-reaction.

Willstätter and his co-workers¹ extracted anthocyanins from flowers and fruits by the use of alcohol diluted with water, and changed them to bright red chlorine derivatives in order to prepare the stable anthocyanidins. After hydrolysis of the glucoside, the anthocyanidins readily went into amyl alcohol in which anthocyanins are insoluble.

Anthocyanidins are divided into three groups—derivatives of pelargonidin, of cyanidin and of delphinidin:



Thus cyanidin is a 4'-hydroxypelargonidin and delphinidin is a 2', 4'-dihydroxypelargonidin.

In addition to these substances, the anthocyan glucosides also contain: Paeonidin, the 3'(?)-monomethyl cyanidin; myrtillidin, the 4'(?)-monomethyl delphinidin; petunidin, isomeric with the preceding one; two dimethyl delphinidins, oenidin and malvidin; and perhaps other derivatives. The ease with which one anthocyanidin may accumulate in place of another one and the lack of characteristic anthocyanins in a given species of plant can be seen in that pelargonidin has been found in the rose-colored strain of corn flowers which usually contain only cyanidin and are blue in color. In the same way, cyanidin has been found in the violet-red races of *Pelargonium* which usually contains only pelargonidin. Other analogous cases are known. The flower color depends on: 1. the content of different anthocyanins, 2. the reaction of the cell sap, and 3. the combination of anthocyanins with the orange-yellow pigments. Moreover, different anthocyanidins do not have the same color tones in all cases. In order to illustrate the character of the natural anthocyanins, the products of hydrolysis of some pigments are given in the following table:

¹ Willstätter, R. und Everest. *Liebig's Ann. d. Chem.* 401: 189. 1913; Willstätter und Nolan. *Ibid.* 408: 1 and 136. 1915; Willstätter und Mallison. *Ibid.* 408: 15 and 147. 1915; Willstätter und Bolton. *Ibid.* 408: 42. 1915; 412: 113 and 136. 1916; Willstätter und Mieg. *Ibid.* 408: 61 and 122. 1915; Willstätter und Zollinger. *Ibid.* 408: 83: 1915; 412: 164 and 195. 1916; Willstätter und K. Martin. *Ibid.* 408: 110. 1915; Willstätter und Burdick. *Ibid.* 412: 149 and 217. 1916; Willstätter und Weil. *Ibid.* 412: 178 and 231. 1916.

² Willstätter und Mallison. *Sitzungsber. preuss. Akad. Wiss. for 1914*, p. 769.

³ Willstätter und Zechmeister. *Ibid.* p. 886.

Plants	Products of anthocyan hydrolysis
<i>Centaurea cyanus</i> , flowers.....	2 mol. glucose + cyanidin
<i>Rosa gallica</i> , flowers.....	The same glucoside
<i>Vaccinium myrtillus</i> , fruit skins.....	1 mol. galactose + myrtillidin
Red <i>Pelargonium</i> , flowers.....	2 mol. glucose + pelargonidin
<i>Delphinium consolida</i> , flowers.....	2 mol. glucose + 1 mol. hydroxybenzoic acid + delphinidin
Wine grapes, skins.....	1 mol. glucose + oenidin
<i>Vaccinium vitis-idaea</i> , skins.....	1 mol. galactose + cyanidin
<i>Althaea rosea</i> , flowers.....	1 mol. glucose + myrtillidin
<i>Malva sylvestris</i> , flowers.....	2 mol. glucose + malvidin
<i>Paeonia</i> , flowers.....	2 mol. glucose + paeonidin
<i>Salvia coccinea</i> }	2 mol. glucose + undetermined components + pelargonidin
<i>Salvia splendens</i> }	
<i>Chrysanthemum indicum</i>	1 mol. glucose + cyanidin
<i>Prunus avium</i>	1 mol. glucose + 1 mol. rhamnose + cyanidin
<i>Prunus spinosa</i>	1 mol. glucose + 1 mol. unknown hexose + cyanidin
<i>Viola tricolor</i>	2 mol. glucose + 1 mol. rhamnose + delphinidin
<i>Petunia hybria</i>	2 mol. glucose + petunidin
<i>Papaver rhoeas</i>	2 anthocyan; one of them yielded 2 mol. glucose + cyanidin (isomeric with the pigment of the corn flower and rose).

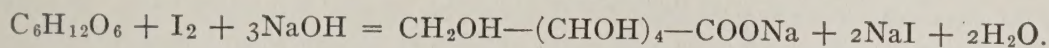
This indicates that cyanidin is the most widely distributed in plants. In conclusion, it should be noted that the physiological importance of the non-sugar components of glucosides can be extremely varied. For example, it is found that the flavone derivatives, according to the latest reports, are very widespread in tropical and alpine plants and play an important rôle as screens to the ultra-violet rays. In plants grown under glass there is a small amount of flavone derivatives. In living plant tissues the flavone derivatives are said to change to anthocyanidins and vice versa.

12. ANALYTICAL METHODS

The simple sugars are detected qualitatively by the preparation of their characteristic derivatives, chiefly the osazones of phenylhydrazine or the hydrazone of substituted phenylhydrazines. The characteristic derivatives or the various sugars have been given above. On account of the high solubility and difficulty of crystallization of the simple sugars, it is recommended that they be isolated for qualitative tests only when the particular sugar is present in considerable quantity and is not mixed with other sugars. The osazones crystallize well from 50% alcohol if pyridine is added by drops during the boiling until the precipitate is dissolved. The analysis of the osazone only indicates whether the sugar is a pentose, hexose, etc. Complete identification requires exact measurement of the melting point of the osazone and its specific rotation.¹ The latter determination can be made still better directly with the purified solution of the sugar. The sugar in the solution is first determined quantitatively by its reduction of Fehling's solution. Then the specific rotation is measured in the polarization apparatus, keeping in mind the result just obtained. The determination of the rotation of the sugar solution is especially important in differentiating between glucose and fructose because both give the same osazone. In addition, ketoses form osazones with methylphenylhydrazine

¹ Neuberg, C. Ber. chem. Ges. 32: 3384. 1899.

much faster than aldoses.¹ The most reliable method of detecting glucose in the presence of fructose is to change the glucose to gluconic acid by oxidation with iodine in alkaline solution; the fructose is not oxidised. This is the iodine method of quantitative determination of grape sugar in the presence of fructose², according to the reaction:



For the rapid recognition of fructose, there is the browning of the solution by boiling in a slightly acid medium, and the following tests:

1. Seliwanow's test.³ Warming fructose solutions with resorcin and hydrochloric acid gives a cherry-red color.

2. Jolles's test.⁴ Fructose is detected in urine by boiling the urine with half its volume of a 20% alcoholic solution of diphenylamine and an equal volume of concentrated hydrochloric acid. A turbidity and red color appears in the presence of fructose. None of these qualitative tests are entirely reliable.

Crystalline di- and trisaccharides are more easily prepared in pure form than the simple sugars. The identification of those which do not possess aldehydic properties consists in determining the specific rotation, the change in rotation on hydrolysis, and the nature of the products formed by hydrolysis. The same methods are used to identify the glucosides. The polysaccharides with large molecules are likewise identified from the cleavage products and the results of enzyme action. Starch can be recognized by observing the starch grains under the microscope and it is possible to determine even the species of plant to which it belongs. Cellulose is identified by insolubility in alkalies and acids and its solubility in Schweizer's reagent. The isolation of cane sugar from plants is facilitated by special methods.⁵

The quantitative determination of sugars is done according to several methods among which the polarization method comes first.⁶ The carbohydrate is determined according to the following formula:

$$C = \frac{\alpha V}{[\alpha]_D l}$$

In this equation C is the concentration of sugar, α is the angle of rotation of the solution, V the volume of the solution, $[\alpha]_D$ the specific rotation of the sugar used and l the length of the tube in the polarimeter. This method is also useful for disaccharides and even for substances with high molecular weights, such as starch and glycogen. On account of the considerable size of the specific rotation of most carbohydrates, the presence of other optically active substances has no great effect.

Pentoses and glycuronic acid are changed to furfural by heating with hydrochloric acid, distilled and furfural precipitated in the distillate with phloroglucin⁶ or barbituric acid.⁷ The precipitate is weighed and the amount of each pentose calculated by the use of a specific, empirically established quotient.

Glucose, invert sugar, fructose, galactose and maltose are determined easily by the reduction of cupric oxide in alkaline solution (Fehling's solution). There are many modifications of this method⁸ including the following two which are especially useful. According to Bertrand,

¹ Neuberg, C. *Ibid.* 35: 959. 1902; however cf. Ofner, R. *Zeitschr. f. physiol. Chem.* 45: 359. 1905.

² Willstätter, R. und G. Schudel. *Ber. chem. Ges.* 51: 780. 1918.

³ Seliwanow, F. *Ber. chem. Ges.* 20: 181. 1887.

⁴ Jolles, A. *Zeitschr. f. physiol. Chem.* 81: 203. 1912.

⁵ Schulze, E. und F. Seliwanow. *Landwirt. Versuchs-Stationen.* 34: 408. 1887. Winterstein, E. *Zeitschr. f. physiol. Chem.* 104: 217. 1919.

⁶ A list of selected methods for the determination of soluble carbohydrates is given by Appleman, C. O. *et. al.* *Plant Physiology.* 2: 195-204. 1927.—*Ed.*

⁷ Kröber. *Jour. f. Landwirtschaft*, p. 355. 1900 and p. 7. 1901.

⁸ Grund. *Zeitschr. f. physiol. Chem.* 35: 111. 1902.

⁸ Soxhlet. *Jour. f. prakt. Chem. n. f.* 21: 227. 1880; Allihn. *Ibid.* 22: 55. 1880; Bang, I. *Biochem. Zeitschr.* 2: 271. 1906; Vernon, H. M. *Jour. of Physiol.* 28: 156. 1902; Pflüger. *Pflüger's Arch. f. ges. Physiol.* 69: 399 and 423. 1898; Bertrand, G. *Bull. Soc. Chim. de France.* 35: 1285. 1906. In all these methods it is necessary to remove proteins and other colloids from the solution.

the precipitated cuprous oxide is dissolved in a solution of ferric sulphate in sulphuric acid and the oxide formed thereby is titrated with permanganate. In gravimetric methods the cuprous oxide is filtered off and weighed either directly (Pflüger's method) or after reduction to metallic copper (Allihn's method).

A very exact quantitative method for fermentable sugars is to split it by yeast.¹ The fermented sugar is calculated from the amount of CO₂ released, according to the equation $C_6H_{12}O_6 = 2C_2H_5OH + 2CO_2$ (subject to empirical corrections). The carbon dioxide is caught in a weighed potash apparatus or in a tube of soda lime.

Starch is determined either from the amount of sugar formed on hydrolysis² (with acidified water under 3-5 atmospheres of pressure), or polarimetrically after dissolving the starch in dilute sulphuric acid.³ The methods designed for animal glycogen⁴ cannot be used for plant glycogen without making changes.⁵ The quantitative determination of cellulose is usually made without purifying it entirely and is therefore not absolutely accurate. The material is digested for several days in a closed vessel of nitric acid in the presence of KClO₃, the undissolved residue treated with ammonia, washed and weighed.⁶ There are several modifications of the determination of "crude fiber," as the chemically impure cellulose is generally called.

13. CARBOHYDRATE TRANSFORMATIONS IN SEED-PLANTS

The change of simple sugars to disaccharides and the reverse hydrolysis of disaccharides in seed-plants is associated with the movement of carbohydrate in the vascular tissues. The most important and common carbohydrates are; first, starch and polysaccharides of the cell wall, secondly, the crystalline disaccharides especially cane sugar and maltose, and thirdly, the simple sugars. Only the latter can diffuse through the plasma membrane and be used for physiological reactions. Cane sugar diffuses very slowly through the outer layer of protoplasm and acts as a reserve material which is dissolved in the cell sap.⁷ The polysaccharides are insoluble reserve materials.

Oftentimes not maltose but cane sugar appears in the cell sap when starch is hydrolyzed. This is not surprising since cane sugar is synthesized after the hydrolysis of the maltose, because glucose readily changes to fructose (p. 238). There are unpublished observations which suggest that glucose and fructose can easily be changed to each other in the organs of the plants.

In Chap. II it was shown that carbohydrates are formed in leaves in the light. In actively functioning leaves an increase in cane sugar and starch as well as in the simple sugars can be proved. In leaves separated from the plant,

¹ Jodlbauer, M. *Zeitschr. f. Ver. d. Zuckerindustrie* for 1888, pp. 308, 313 and 346. Determination of lactose: Hildt, E. *Compt. rend.* **167**: 756. 1918. Determination of maltose and lactose: Legrand M. *Ibid.* **172**: 602. 1921.

² Franke, G. *Zeitschr. f. physiol. Chem.* **7**: 510. 1883.

³ Lintner, C. J. *Zeitschr. f. ges. Brauwesen.* **30**: 109. 1907; *Idem.* *Zeitschr. f. Untersuch. f. Nahrungs. und Genussmittel.* **14**: 205. 1907; **16**: 509. 1908; Ewers, E. *Zeitschr. f. öffentl. Chem.* **14**: 8 and 150. 1908; **15**: 8. 1909. [For comparative work in the determination of small quantities of starch in plant tissues see Bish. *Biochem. Jour.* **23**: 31-34. 1929. For methods used for various polysaccharides see Appleman, C. O. *et al.* *Plant Physiology.* **2**: 91-97. 1927.]

⁴ Pflüger. *Pflüger's Arch. f. ges. Physiol.* **229**: 374. 1909.

⁵ Salkowski, E. *Zeitschr. f. physiol. Chem.* **92**: 75. 1914. Cf. the biochemical determination of glycogen by Mayer, P. *Biochem. Zeitschr.* **136**: 487. 1923.

⁶ Schulze, Fr. *Beiträge zur Kenntnis des Lignins.* 1856; Henneberg. *Liebig's Ann. d. Chemie.* **146**: 130. 1868. Determination of pure cellulose: Kiesel, A. und Semiganowsky. *Ber. chem. Ges.* **60**: 333. 1927.

⁷ The recently published work of Mason, T. G. (*Ann. Bot.* **42**: 571-636. 1928) on the transport of carbohydrates in the cotton plant indicates that cane sugar may be the form in which carbohydrates pass downward in the phloem tissues of the stem.—Ed.

a large part of the carbon assimilated goes into the carbohydrates of the cell walls.¹

Along with the formation of carbohydrates in leaves, there is also a rapid drainage of them into the stem, as Sachs pointed out long ago.² Starch and soluble saccharides are only temporarily formed in leaves; this is because the concentration of sugar becomes very high during active photosynthesis; hence the action of the enzymes is synthetic.³ The temporary cessation of photosynthesis is enough to start the hydrolysis of starch. For example, starch starts to dissolve when the leaves wilt.⁴ In the leaves there are the enzymes amylase⁵ and invertase⁶ which transform the carbohydrates. In some *Compositae* starch is replaced by inulin.⁷ In other plants, especially in the *Oleaceae*, there is, in addition to the sugars, mannite⁸ which serves as material for the building of starch (of course after oxidation to sugar). Low temperatures retard the synthetic processes of forming the insoluble polysaccharides and the sugar content of the leaves increases.⁹

Since neither sugar nor polysaccharides remain long in the leaf, but pass into the stem, there is a certain equilibrium between the formation of carbohydrate and its removal. The most recent investigations of the carbohydrate content of leaves at different periods of growth have given unexpected results.¹⁰ It was found that the leaves which fall in the autumn contain large amounts of simple sugars. They are often as rich in sugar as young leaves. In the same way, large amounts of soluble carbohydrates have been found in annual plants at the end of their existence. In early summer large carbohydrates accumulate in the leaves while soluble sugars predominate in autumn. The following table from Michel-Durand (*loc. cit.*) illustrates the carbohydrate content of 100 birch leaves at various stages of growth.

	Soluble sugar g.	Starch and hemicellu- lose g.	Glucosides g.	Total g.
May 9 green leaves.....	2.87	2.98	6.19	12.04
Sept. 11 green leaves.....	5.22	4.91	10.48	20.61
Oct. 28 yellow leaves.....	5.18	4.67	7.91	17.76
Oct. 30 fallen leaves.....	3.00	4.97	6.68	15.65

¹ Körösy, K. v. Zeitschr. f. physiol. Chem. 86: 368. 1913.

² Sachs, J. Arb. d. bot. Inst. zu Würzburg. 3: 1. 1884.

³ Schimper. Bot. Zeit. 43: 769. 1885. On the periodic fluctuation of the amount of carbohydrate in leaves cf. Gola. Atti dell' ist. bot. di Padova for 1923, p. 71; for the same in fruits: Abbott, O. Bot. Gaz. 76: 167. 1923.

⁴ Horn, F. Bot. Arch. 3: 137. 1923.

⁵ Brasse, L. Compt. rend. 99: 878. 1884; Brown, H. T. and Morris. Jour. Chem. Soc. (London) 60: 604. 1893.

⁶ Kastle and M. E. Clark. Amer. Chem. Jour. 30: 422. 1903.

⁷ Grafe, V. und Youk. Biochem. Zeitschr. 47: 320. 1912; Colin. Rev. gén. bot. 31: 75. 1919.

⁸ Vogel, A. Schweigg. Jour. 37: 365. 1823; Maquenne, L. Les sucres. 1900.

⁹ Lidforss. Bot. Centralbl. 68: 33. 1896; Czapek, F. Ber. bot. Ges. 19: 120. 1901.

¹⁰ Michel-Durand, E. Rev. gén. bot. 30: 337. 1918; Combes, R. et D. Kohler. Compt. rend. 175: 590. 1922.

This shows that plants lose much sugar in autumn by the shedding of the leaves.^s The same thing occurs at the death of annual plants.

The carbohydrates which pass from the leaves to other parts of plants serve different purposes which can be divided into four categories:

1. Processes for the release of energy.
2. Chemical transformations into other substances needed by the plant.
3. Building of new organs and tissues.
4. Storage of reserve substances.

Most of the carbohydrate is used to release energy. Sugar is consumed in respiration and provides the plant with the energy that corresponds to the energy from the combustion of heating materials in factories. These biochemical processes will be considered in Chap. VIII. The synthesis of other biologically important substances from sugar will be described in the next chapter. From the chemical standpoint, the building of new organs by growth is due chiefly to the synthesis of cell wall materials and of the proteins which are the principal material from which protoplasm is made. The synthesis of proteins will be discussed later. As regards the synthesis of cell wall materials, the mechanism of this process is not yet clear but it seems to be a reversal of the processes by which the cell walls are dissolved by cytase and other enzymes which break down cell walls.

The storage of reserve carbohydrates and the consumption of these reserves is a very widespread phenomenon among the seed-plants. There are special places for the stored foods. In the perennial herbs, the rhizomes, tubers and bulbs are often containers of reserve substances while in the trees the organic material for the growth of leaves in the spring is stored in the autumn in the living cells of the woody cylinder. The commonest place for reserves is in seeds where carbohydrates or fats plus proteins are deposited either in the endosperm or in the cotyledons. In both seeds and underground reservoirs of reserve foods, starch and soluble sugars can be found at any time. The proportion of these products varies at different periods in the life of the plant and at different times of year. Only grape sugar can be detected from the cleavage of hemicelluloses (mannans and galactans) because mannose and galactose change very rapidly to glucose. The presence of reserve food containers explains the phenomenon of physiological periodicity. During the latent period, *i.e.*, in winter, it is usually almost impossible to bring about the cleavage and assimilation of starch reserves, even under favorable conditions.^t Large amounts of

^s When the corolla falls from the flower there is a loss of carbohydrates; *cf.* Iwanow, S. Ber. bot. Ges. 45: 582-587. 1927.—*Ed.*

^t This is the problem of breaking the rest period, a problem that is very important for the nurseryman and other growers of plants. Many treatments have been devised and shown to be more or less effective. For a chemical analysis of the effects of breaking the rest period of potato tubers by means of ethylene chlorhydrin ($\text{ClCH}_2\text{CH}_2\text{OH}$) or sodium thiocyanate (NaSCN), see Denny, F. E. Amer. Jour. Bot. 16: 326-337. 1929; 17: 806-817. 1930. The sucrose content is found to increase at the expense of the starch. One of the most effective methods used in connection with seeds which show definite periods of dormancy is that of stratification which involves storage of the seed at certain temperatures for several weeks. Many of the most important results in this field have appeared in the Amer. Jour. of Botany and other American journals during the past few years. Most of this work has been done by workers at the Boyce Thompson Institute at Yonkers, N. Y.—*Ed.*

enzymes are formed when the seeds begin to germinate¹ and the polysaccharides are rapidly consumed and the endosperm and other reserve food containers are emptied. The following table from André,² showing the use of starch by *Phaseolus multiflorus*, gives the decrease in the starch supply during germination:

June 26.....	62.07 g. starch	July 11.....	20.18 g. starch
July 5.....	52.40 g. starch	July 15.....	16.40 g. starch
July 8.....	34.49 g. starch	July 19.....	14.61 g. starch

Amylase is formed in the seedling as well as in the endosperm and the former may even contain more, especially in the scutellum.³ It is worth noticing that considerable amounts of cane sugar are always formed in the hydrolysis of starch⁴ as is also the case in the leaves (see on p. 256). Germinating seeds always contain invertase⁵ and maltase.⁶

There have been interesting experiments in which the endosperm is emptied in the absence of the seedling. The latter is replaced by a column of gypsum which has its lower end immersed in water. The cleavage products of the polysaccharides diffuse through the column into the water and this prevents retardation of starch hydrolysis by reason of the accumulation of products of the reaction.⁷ The endosperm entirely empties under these conditions. The same thing occurs if a living or a dead endosperm of another seed or even of a different species of plant is connected to the seedling.⁸ From all these experiments it can hardly be concluded that endosperm is a dead tissue. It must not be forgotten that many observations have proved the capacity of amylase to diffuse through parchment and even through protoplasm.⁹

The reserve material deposited in cell walls as well as starch is used in seed germination.¹⁰ The work of Brown and Morris¹¹ shows that enzymes which attack the polysaccharides of the cell wall are formed in large amounts, along with diastase, in the germinating scutellum. Accurate analyses¹² show that in the germination of a legume (*Lupinus luteus*), nine-tenths of the polysaccharides

¹ Effront. Compt. rend. 141: 662. 1905.

² André. Ibid. 130: 728. 1900.

³ Brown and Morris. Jour. Chem. Soc. (London) 57: 508. 1890; Linz. Jahrb. wiss. Bot. 29: 267 1896.

⁴ Kühnemann, G. Ber. chem. Ges. 8: 202 and 787. 1875; Brown and Morris. loc. cit., p. 459; Schulze, E. Ber. bot. Ges. 7: 280. 1889; O'Sullivan. Jour. Chem. Soc. (London) 53: 55. 1886; and others.

⁵ Brown and Heron. Jour. Chem. Soc. (London) 35: 609. 1879; Vandeveld. Biochem. Zeitschr. 28: 131. 1910; and others.

⁶ Beijerinck. Centralbl. f. Bakt. (II) 1: 329. 1895; Huerre, R. Compt. rend. 148: 1526. 1909; and many others.

⁷ Hansteen. Flora. Erg.-Bd. 79: 419. 1894; Puriewitsch. Investigations of the emptying of reserve food reservoirs. 1897 (Russian).

⁸ Brown and Morris. Jour. Chem. Soc. (London) 57: 458. 1890; Brown and Escombe. Proc. Roy. Soc. London. 63: 3. 1898; Stingl. Flora. 97: 308. 1907.

⁹ Krabbe. Jahrb. wiss. Bot. 21: 4. 1890; Richter, A. Jour. russ. bot. Ges. 2: 56. 1917 (Russian); and others. [The association of amylase with mitochondria and the correlation of their migrations with the hydrolysis of starch in the endosperm cells into which they pass, has been reported by Horning and Petrie. Proc. Roy. Soc. London (B) 102: 188-206. 1927.]

¹⁰ Mitscherlich. Liebig's Ann. d. Chem. 75: 305. 1850.

¹¹ Brown and Morris. Jour. Chem. Soc. (London) 75: 548. 1890.

¹² Schulze, E. und Steiger. Landwirt. Versuchs-Stationen. 36: 391. 1889; Schulze, E. Ber. bot. Ges. 14: 66. 1896.

are dissolved and on hydrolysis they yield 1 part of grape sugar and 24 parts of galactans.

Unfortunately, the reverse process of storage of polysaccharides in ripening seeds has been investigated only in spots. That the law of mass action is very significant in determining the enzyme reactions of seeds is evident from the foregoing experiments on the emptying of the reserve food containers through columns of gypsum which conduct sugar solutions to the water.¹ The cotyledons of *Phaseolus* and *Lupinus* were emptied into the water while the starch poured into the sugar solution. It is interesting that amylocoagulase has been found in seeds. This enzyme causes condensation of molecular complexes in addition to forming insoluble starch grains from soluble starch.² Cane sugar also appears as an intermediate stage in the formation of starch in ripening seeds.³

In general, the same process takes place in underground reserve food containers as in seeds. Much starch and hemicellulose is deposited there and inulin occurs in various *Compositae*. Enormous amounts of cane sugar are often stored there and at a certain time of year there is a breaking down of polysaccharides by means of large amounts of enzyme. Most interesting—especially in the potato—is the phenomenon of transformation of starch to sugar at the low temperatures of the winter rest period. It has been proved beyond doubt⁴ that this process is brought about only by temperatures below $+9^{\circ}\text{C}$. The reverse process takes place in a warm place; at $20-30^{\circ}$, 70% of the sugar is changed back into starch. Cane sugar also appears in this case and the whole process is a combination of hydrolysis and synthesis. From recent information⁵ it is concluded that an abundant supply of potassium salts increases the sugar content in both seeds and underground reservoirs.

The decomposition and synthesis of glucosides have been very little studied. According to the view of many investigators, hydrocyanic acid undergoes a continuous metamorphosis in the plant body and the content of nitrile glucosides fluctuates accordingly.⁶ Amygdalin is used in the germination of almond seeds and the hydrocyanic acid of this glucoside is also consumed.⁷ On page 234 it was pointed out that hydrocyanic acid may be important in the synthetic processes. The polyvalent phenols and other easily oxidized substances which are often contained in glucosides act as "respiratory chromogens" (Chap. VIII)

¹ Puriewitsch. *loc. cit.*; Idem. *Jahrb. wiss. Bot.* 31: 69: 1898.

² Maquenne, L. *Compt. rend.* 137: 88 and 797. 1903; 138: 49, 213 and 375. 1904; Wolff et Fernbach. *Ibid.* 137: 718. 1903; 138: 819. 1904; 139: 1217. 1904; 140: 95 and 1547. 1905; 144: 645. 1907; Idem. *Ann. Inst. Pasteur*, Vol. 18. 1904; Roux, E. *Compt. rend.* 140: 943 and 1259. 1905; 142: 95. 1906.

³ Frerichs und Rodenberg. *Arch. f. Pharmazie.* 243: 675. 1905.

⁴ Müller-Thurgau. *Landwirt. Jahrb.* 11: 744. 1882; 14: 909. 1885; Idem. *Flora.* 101: 309. 1910; 104: 387. 1912.

⁵ Stoklasa, J. *Zeitschr. f. landwirt. Versuchs. Öster.* 15: 711. 1912; Idem. *Biochem. Zeitschr.* 73: 107. 1916; 82: 310. 1917; De Plato, G. *Ann. della staz. chim. agric. sper.* (2) 3: 195. 1909.

⁶ De Plato. *Staz. sperim. agron.* 46: 449. 1911; Ravenna e Vecchi. *Atti d. Reale accad. dei Lincei, rendiconto.* 20: 491. 1911; cf. also Jorissen, A. *Bull. Acad. Roy. Belgique for* 1913, p. 1202; Guignard, L. *Compt. rend.* 147: 1023. 1908; De Jong, A. W. K. *Ann. jardin bot. des Buitenzorg.* 22: 1. 1908.

⁷ Korsakowa, M. *Ber. milchwirt. Inst. Wologda.* 2: 3. 1922; Ravenna e Zamorani. *Ann. di botanica.* 8: 51. 1910; Idem. *Atti d. Reale accad. dei Lincei, rendiconto.* 19: 356. 1910.

in the oxidation processes, according to Palladin's view.¹ Palladin attributes an analogous importance to some anthocyanins and indican glucosides.² Phloroglucin, a common component of many glucosides, may serve as material for the synthesis of tannins.³

Exhaustive studies of the feeding of seed-plants with glucosides in *pure cultures*⁴ have shown that the poisonous glucosides are not toxic to the plants from which they are obtained. It seems that glucosides are not always consumed in plants; for example, the saponins are not utilized in germination⁵ and their rôle in plants remains unknown. However, it is quite possible that the "mobilization," *i.e.*, the hydrolytic cleavage of glucosides, does not depend on the renewing of life in the spring and on the processes of germination, but is due in the first place to the consumption of the non-sugar components of the glucosides.

14. CARBOHYDRATE TRANSFORMATIONS IN THE LOWER PLANTS

Under favorable conditions there is a considerable deposition of starch in algae that are capable of photosynthesis. It is the same in mosses. Moreover, certain uninvestigated carbohydrates, such as leucosin, anabenin, etc., have been described from the lower algae. Careful study may show that they are identical with known polysaccharides. The formation of carbohydrates with high molecular weights in the algae probably depends on the high content of sugar in their cell sap during rapid photosynthesis, especially to prevent the development of an excessive osmotic pressure.

There are peculiar transformations of carbohydrates in the fungi. In the gill fungi, simple sugars are often replaced by the hexavalent alcohol mannite, cane sugar by the disaccharide trehalose, and starch by another amorphous polysaccharide, glycogen. The wide distribution of mannite in the fungi has been known for a long time.⁶ In the mushrooms we often find a large content of mannite but not a trace of the simple sugars and a considerable hydrolysis of mannite under the influence of specific enzymes, can often be found even in the expressed juice of fungi.⁷ It is an interesting fact that mannite is rapidly formed from trehalose when the fungi are removed from the soil and allowed to dry slowly.⁸ Trehalose is often utilized directly to provide energy.⁹ For example, in one of Bourquelot's experiments 20 grams of trehalose were found

¹ Palladin, W. Ber. Russ. Akad. Wiss. for 1908, pp. 447 and 977; 1909: 371 and 459; 1912: 438; 1913: 93 (Russian); Idem. Biochem. Zeitschr. 18: 151. 1909; 27: 442. 1909; Idem. Rev. gén. bot. 23: 225. 1911; Idem. Ber. bot. Ges. 26a: 125, 378 and 389. 1908; 27: 101. 1909; 30: 104. 1912; Idem. Zeitschr. f. Gärungsphysiol. 1: 91. 1912; Palladin, W. und S. Lwov. Ibid. 2: 326. 1913.

² Palladin, W. *loc. cit.*; Walter, O. Bot. Jour. Naturforscherges. in Petersburg. 3: 199. 1908 (Russian).

³ Waage, W. Ber. bot. Ges. 8: 250. 1890.

⁴ Combes, R. Rev. gén. bot. 29: 321 and 353. 1917; 30: 5. 1918.

⁵ Korsakowa, M. Compt. rend. 155: 844 and 1162. 1912; Idem. Rev. gén. bot. 26: 225. 1914; Moycho, V. Ibid. 32: 449. 1920.

⁶ Muntz, A. Ann. chim. et physique. 8: 56. 1876.

⁷ Kostytschew, S. Zeitschr. f. physiol. Chem. 65: 350. 1910.

⁸ Bourquelot, E. Compt. rend. 108: 568. 1889; 111: 534. 1890; Idem. Bull. soc. mycol. 5: 34 and 132. 1889; 6: 150 and 185. 1891; 7: 50. 1891; 18: 13. 1892. Trehalose has also been found in the molds: Bourquelot, E. *loc. cit.*; Idem. Bull. soc. mycol. 9: 11 and 189. 1893.

⁹ Iwanow, N. N. Biochem. Zeitschr. 135: 1. 1923.

in 4 kg. of *Agaricus piperatus* and after 5 hours there was not a trace of it left. Glycogen is formed and broken down just as rapidly. Glycogen is contained in the molds as well as in the gill fungi; in yeast the glycogen content often reaches 25–40% of the dry substance¹ but it all disappears in 3–4 hours if the yeast is dried slowly.² In emergencies, the reserve substances in the cell walls of fungi³ are rapidly absorbed. In many fungi the nitrogen-free reserve material is stored in the form of a mucilage, the chemical nature of which is undetermined.⁴

15. THE GENERAL NATURE OF PROTEINS

Proteins are colloidal substances of high molecular weight which are formed of nitrogen and sulphur and often phosphorus, in addition to carbon, hydrogen and oxygen. They occur in large quantities in all living cells in that they make up more than half the dry weight of protoplasm. Moreover, they are often stored as reserve foods. In the vascular tissues they are found in the cell sap of the sieve tubes and latex vessels. The rapid progress of the chemistry of proteins during recent years teaches us that they—contrary to the older, unproved ideas—are not unstable compounds which readily enter into various reactions and that they can hardly serve as active factors in vital phenomena. In physiological respects, it is not so much the chemical as the physical properties of proteins that are important. Thanks to these characteristics, they form a very suitable substrate for vital processes and a heterogeneous medium which readily changes its reaction and electrical charge and thereby favors first the synthetic and then the hydrolytic reactions. Hence the conception of proteins as the only living substance in preference to the other chemical substances was a wholly unfounded idea.

In physical respects the proteins are reckoned in the group of hydrophile colloids (p. 11). They do not diffuse through membranes, their solutions are opalescent and on shaking they form a froth which is not easily dissolved. The internal friction of protein solutions is very high.⁵ They often filter with great difficulty. Nevertheless, the proteins form partly ionized salts which form true solutions.⁶ By changes in the reaction of the medium and by other effects, the proteins readily take on the properties of hydrophobe colloids and exhibit the characteristic phenomenon of cataphoresis when an electric current is passed through them. From these facts it is evident that the solubility of proteins depends to a large extent on the external conditions. As another indication of the intermediate position of the proteins between typical colloids and electrolytes, we have the capacity of proteins to take the form of rather irregular crystals under certain conditions. Under natural conditions such crystals often

¹ Pavy, F. W. and Bywaters. Jour. of Physiol. 36: 149. 1907; Henneberg. Zeitschr. f. Spiritusindustrie. 33: 242. 1910.

² Buchner, E. und Mitscherlich. Zeitschr. f. physiol. Chem. 47: 554. 1904.

³ Winterstein, E. Ber. chem. Ges. 28: 774. 1895; Dox, A. W. and Neidig. Jour. Biol. Chem. 9: 267. 1911.

⁴ Zellner, J. Monatsh. f. Chem. 27: 281. 1906; Foëx, E. Compt. rend. 155: 661. 1912; and others.

⁵ Sackur, O. Zeitschr. f. physikal. Chem. 41: 679. 1902.

⁶ Bugarsky und Liebermann. Pflüger's Arch. f. ges. Physiol. 72: 51. 1898.

form in the reserve food reservoirs (in the aleurone grains^u) and in the nuclei of some plants. They have also been prepared from albumin, haemoglobin and a few other animal proteins.¹ However, it is only very recently² that crystals of pure protein have been obtained because the crystals take up impurities by adsorption.

Modification of the properties of proteins under the influence of various effects is called coagulation and denaturing. Coagulation makes the protein insoluble and the same thing often happens in denaturing but in other cases the protein remains in solution but no longer has its natural properties. Thus coagulation seems to be a special case of denaturing which involves chiefly a loss of capacity to change readily from the gel to the sol condition and vice versa without change in its characteristic properties.

Denaturing of proteins is best known for the case of heating the solution; the protein is precipitated in flakes which do not dissolve again when cooled. Complete precipitation can be had in a weakly acid solution. If too much acid is added, part of the denatured protein redissolves on account of loss of electrical charge (p. 11). Other denaturing effects to be mentioned are: treatment with acids and alkalis (in the latter case the alkali salt of the denatured protein frequently goes into solution); excess of alcohol, acetone, chloroform and similar substances; likewise the addition of salts of the heavy metals, such as lead, copper, silver, mercury, etc., which form insoluble compounds with the proteins. In all these cases, the denaturing is not instantaneous but comes in a very short time.³ Even a persistent shaking of the protein solution and the effect of adsorbed substances, like finely divided charcoal, loam, etc., may have a denaturing effect.⁴

The only known methods of removing unchanged proteins from solution are to salt them out and—for proteins insoluble in pure water—dialysis. In the consideration of the physiological importance of the effect of ions on colloids, the question of salting out was discussed from the theoretical standpoint in Chaps. I and V. It is sufficient here to point out the practical usage. The protein that is thrown out of solution by the addition of salt is redissolved if the salt is removed by dialysis or if the concentration of salt is lowered by dilution with water. This makes practically no change in the properties of the protein which is denatured only on long standing.

Different salts do not precipitate the protein equally well. Zinc sulphate and ammonium sulphate have the strongest action.⁵ The latter is used for the

^u The view has been expressed by Peklo and summarized in a paper by Jodidi, S. L. and J. Peklo (Jour. Agr. Res. 38: 69-91. 1929) to the effect that the deposits of proteins in aleurone grains are due to the presence of a fungus which lives as a symbiont in cereal seeds. Some of these fungi have been isolated.—Ed.

¹ Hofmeister. Zeitschr. f. physiol. Chem. 14: 163. 1889; Schulz, F. N. Die Krystallisation von Eiweissstoffen. 1901.

² Sørensen, S. P. L. und M. Höyrup. Zeitschr. f. physiol. Chem. 103: 15 and 104. 1918.

³ Galeotti, G. Zeitschr. f. physiol. Chem. 40: 492. 1903; Spiro, K. Hofmeister's Beitr. z. chem. Physiol. und Path. 4: 300. 1903.

⁴ Ramsden, W. Dubois-Archiv for 1894, p. 517; Hermann, L. Pflüger's Arch. f. ges. Physiol. 26: 442. 1871.

⁵ Kühne, W. und R. H. Chittenden. Zeitschr. f. Biol. 20: 11. 1884; 22: 423. 1886; Kühne, W. Verhand. d. Heidelberg natur-med. Ver. n.s. 3: 286. 1885.

fractionation of proteins by salting out.¹ The concentration of salt which throws a given protein out of solution is said to be just as characteristic for these colloidal substances as the solubility is for crystalline substances.²

Determinations of molecular weights of proteins by physical methods have yielded marked discrepancies up to recently, because it has been impossible to remove all the mineral salts adsorbed to the proteins. Of course, the presence of these salts has a strong influence on the determination of the molecular weight and fluctuations of 150% have been found in the work of different writers. Still less reliable are the experiments to measure the molecular weight of proteins by chemical means, such as by introducing a halogen or the nitro group into the protein molecule or by methylation of proteins, because it is left undetermined how many of these groups become attached to one molecule of protein.

Only very recently have the thorough experiments of Sørensen and his co-workers³ made it possible to undertake dialysis of proteins under conditions which permit the complete separation of protein from the mineral impurities. The determination of the molecular weight of a protein entirely free from ash, on the basis of the osmotic pressure of its solutions, yielded the enormous number of 34,000 with 380 atoms of nitrogen per molecule.⁴ Previous investigations had given lower values—5,000 to 15,000. It will be considered later that proteins, like starch and other polysaccharides, may not be chemical units but are to be regarded only as a specially dispersed condition of less complicated molecular complexes. If this is so, the value of determining the "molecular weight" of different proteins is very problematical.

It is impossible to determine even the minimum empirical formulae of proteins by analysis of the elements because the analytical errors correspond to more than one atom of the various elements. Moreover, it must be considered that neither the structure nor the chemical nature of large molecules is given by empirical formulae. The composition of all the proteins with respect to their elements is surprisingly alike although the constitution of the separate proteins exhibits large differences. Both the relatively simple reserve proteins of seeds of plants and the proteins of the blood and the sexual elements of the higher animals contain about 53–54% C, 7% H, 16% N and seldom over 2% S. As typical ampholytes the proteins yield salts with both acids and bases, salts which are more dissociated in solution than the free protein molecule and they undoubtedly also occur under natural conditions. Protein salts are found in the reserve substances of various seeds. Some proteins have predominantly acid properties and others are basic, due to the chemical nature of the single structural units of the protein molecule.^v

¹ Hofmeister, F. *Arch. f. exp. Path.* 24: 247. 1887; 25: 1. 1888; Lewith. *Ibid.* 24: 1. 1887; Effront. *Moniteur Sci.* 16: 241. 1902.

² However, the concentration actually seems to be less constant than was earlier believed; cf. Osborne and Harris. *Jour. Amer. Chem. Soc.* 25: 837. 1903; *Idem.* *Amer. Jour. Physiol.* 13: 436. 1905.

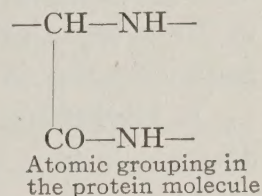
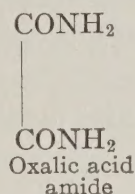
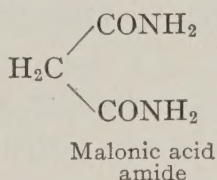
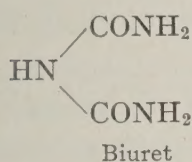
³ Sørensen, S. P. L. und M. Höyrup. *Med. frå Carlsberg lab.* 12: 1. 1917; *Idem.* *Zeitschr. f. physiol. Chem.* 103: 15 and 104. 1918.

⁴ Sørensen, S. P. L. *Zeitschr. f. physiol. Chem.* 106: 1. 1919.

^v A summary of recent advances in the determination of the structure of proteins was given by Klarman. *Chem. Rev.* 4: 51–107. 1927. This review is based largely on Abderhalden's work which indicates that proteins consist largely of cyclic compounds. A new conception of protein structure has ap-

All proteins are optically active and are always levorotatory. The specific rotation has been measured for a few well purified proteins.¹ For edestin of hemp seeds $[\alpha]_D = -41.3^\circ$, for legumin of bean seeds $[\alpha]_D = -41.1^\circ$, and for gliadin of wheat $[\alpha]_D = -92.3^\circ$.

Various colorimetric tests for proteins have been known for a long time. Among them the so-called biuret test is reliable: a violet coloration is obtained by adding a couple of drops of a dilute solution of copper sulphate and an excess of lye to an aqueous solution of protein. Plant proteins and all peptones (see below) yield a red color with a tinge of violet. This test is characteristic for all substances with one of the following atomic groupings:²



Since of all these substances, only proteins occur in the plant cell, the test is reliable for the detection of these substances. It gives negative results with the crystalline products of total hydrolysis of protein. On the contrary, the other supposed "protein tests" are actually tests for the separate amino acids, the structural units of the protein molecule, and therefore yield negative results with proteins in which the given amino acid is lacking. Among these tests the following are useful:

1. *Millon's Test*.—Boiling the protein with a solution of mercuric oxide in an excess of nitric acid forms a coagulum of flesh-red color. This test depends on the presence of the amino acid tyrosine and tyrosine-free proteins gives a negative test.

2. *Xanthoproteic Reaction*.—Heating protein with concentrated nitric acid produces a nitration of the aromatic rings in the protein molecule and a yellow color results.³ The addition of ammonia changes the color to orange.

3. *Adamkiewicz's Test*.—A violet color is obtained by adding glyoxylic acid (prepared by reduction of oxalic acid by sodium amalgam) and an excess of concentrated sulphuric acid to an aqueous solution of protein.⁴ This test (and the one following) is due to the presence of the amino acid tryptophane.

4. *Neubauer's Test*.—A couple of drops of a solution of dimethylaminobenzaldehyde in 10% sulphuric acid gives a reddish violet color with aqueous solutions of protein in the presence of concentrated sulphuric acid.⁵

5. *The Molisch Test*.—The addition of two drops of alcoholic α -naphthol and 1–2 volumes of concentrated sulphuric acid to a solution of protein gives a violet color. This is a furfural reaction. It gives a positive test only with proteins which contain a carbohydrate group, which is not always the case.

6. *Lead Sulphide Test*.—Boiling a protein with a lead salt in the presence of an excess of hydroxide gives a black precipitate of lead sulphide.

peared as the result of proof of the *zwitterion* constitution of amino acids, polypeptides and proteins. This idea was first advanced by Adams (J. Amer. Chem. Soc. 38: 1503, 1916) and Bjerrum (Z. physikal. Chem. 104: 147, 1923) and recently confirmed and augmented by L. J. Harris. Biochem. Jour. 24: 1080–1097, 1930. The constitution of an amino acid is shown to be $^+\text{NH}_3-\text{R}-\text{COO}^-$ in place of the classical $\text{NH}_2-\text{R}-\text{COOH}$.—Ed.

¹ Osborne, T. B. and Harris. Jour. Amer. Chem. Soc. 25: 842. 1903.

² Schiff, H. Ber. chem. Ges. 29: 298. 1896; Idem. Liebig's Ann. d. Chem. 299: 286. 1897; 319: 300. 1901.

³ Salkowski, E. Zeitschr. f. physiol. Chem. 12: 215. 1887; v. Fürth. Diss. Strassburg. 1899; Rohde. Zeitschr. f. physiol. Chem. 44: 161. 1905.

⁴ Hopkins and Cole. Proc. Roy. Soc. London. 68: 21. 1901; Idem. Jour. of Physiol. 27: 418. 1901; Cole. Ibid. 30: 311. 1904; Voisenet tries to show that the color comes from formaldehyde rather than from glyoxylic acid. Cf. Voisenet. Compt. rend. 166: 789. 1918.

⁵ Neubauer. Sitzungsber. d. Ges. f. Morph. und Physiol., München. 1903; Rohde. loc. cit.

The proteins are divided into several groups. The primary distinction is between the simple proteins and the complicated proteins or proteids. The latter are unions of proteins with other molecular complexes. Glucoproteids are the proteins which contain a carbohydrate group. Proteins in which there is a nucleic acid complex are called nucleoproteids. [The present tendency, however, is to avoid the term "proteid" and to speak of glucoproteins and nucleoproteins.]

The simple proteins are usually divided into the following groups:

1. **Albumins**, which are soluble in pure water and are salted out by saturation of their solution with ammonium sulphate. Only small amounts of these proteins occur in plants.

2. **Globulins**, which are insoluble in pure water and are soluble only in dilute solutions of the neutral salts. They are salted out by half-saturation of their solutions with ammonium sulphate and can thereby be separated from the albumins. Many reserve proteins of plants belong in the globulin group.

3. **Prolamines** are reserve proteins which are soluble in 70% alcohol but not in pure water or in salt solutions.

4. **Glutelins** are reserve proteins which are insoluble in water, salt solutions and alcohol. They are soluble only in dilute alkalies.

5. **Protamines** and **histones** differ markedly from the other proteins in the simplicity of their composition, the high content of nitrogen (17.6–19.8% in histones and 25–30% in protamines) and their basic properties,¹ since all other proteins have the properties of weak acids. Protamines and histones are important in fish semen. They have not been found in plants.²

It is evident that the principal plant proteins are found in the globulin group. Of course, this applies only to the reserve proteins since the proteins in the protoplasm and nucleus are chiefly complicated nucleoproteids.

The foregoing classification of proteins is only partly rational. There is a chemical distinction between proteids and proteins but the albumins and globulins are separated only on the basis of unlike solubility and salting out capacity. However, it will be seen from the recent investigations described on p. 285 that these properties are not a sufficient criterion because they do not allow for even an approximation of the value of the molecular weight. The correct chemical system of plant proteins is being worked out by Osborne and his co-workers on the basis of the various cleavage products of the single proteins. This great work is still far from finished. The results already achieved will be considered later.

16. THE PRODUCTS OF TOTAL HYDROLYSIS OF THE SIMPLE PROTEINS

It is evident from the foregoing statements that we can hope to determine the constitution of proteins only by a roundabout way. It will first be necessary to perform a complete hydrolysis of the proteins and to study the crystalline

¹ Kossel, A. und H. Pringle. *Zeitschr. f. physiol. Chem.* 49: 307. 1906; Kossel, A. und F. Kutscher. *Ibid.* 31: 188. 1900; Kossel, A. *Ibid.* 22: 178. 1897; 25: 166. 1898; Kossel, A. und A. Mathews. *Ibid.* 25: 190. 1908.

² N. N. Iwanoff has isolated from *Lycoperdon* a substance suggestive of histopeptone but not very rich in nitrogen. Cf. Iwanoff, N. N. *Mitt. d. russ. Akad. d. Wiss. for 1918*, page 397.

cleavage products. Then we must try to synthesize the protein molecule from these cleavage products. The use of this method for chlorophyll was described in Chap. II and there it was pointed out that the method is sound. The hydrolysis of protein by the usual methods does differ from Willstätter's splitting of chlorophyll in that most of the protein molecule is decomposed at once into different kinds of cleavage products. It is only recently that methods have been found for the gradual splitting of protein. This will be discussed later. We shall first consider total hydrolysis.

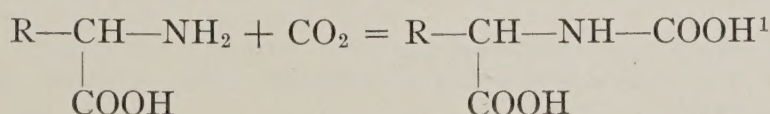
A total hydrolysis of protein to the crystalline cleavage products is effected by prolonged boiling with strong mineral acids. It is generally boiled in a reflux condenser either 6 hours with fuming hydrochloric acid or 16 hours with 25% sulphuric acid. It is quite possible that some labile cleavage products are destroyed by such treatment but about 80% of the nitrogen in protein is recovered in the form of the so-called amino acids. For this reason it is assumed that amino acids are the foundation of the protein molecule.

Amino acids are amphoteric substances which form salts with both acids and bases. They are marked by their resistance to strong acids on long boiling as well as by their high melting points. It is an important fact that the amino group in all natural amino acids is adjacent to the carboxyl group and is therefore in the alpha position. For this reason the difference between the amino acids depends on the unlike structure of the rest of the carbon framework and the general formula for natural amino acids is $R-CH(NH_2)-COOH$. R can be simply a hydrogen atom or a carbon chain which may be very complicated in its structure. Thus there are aromatic rings and other amino and carboxyl groups in some amino acids. The amino acids which include two or more basic groups for each carboxyl group have well defined basic properties and are called diamino acids. On the contrary, there are amino acids with two carboxyl groups for each amino group and these have acid properties.

With the exception of the first member of the series of amino acids—glycine—all the other amino acids are optically active. Only certain optical isomers of amino acids have been found in all the animal and plant proteins.

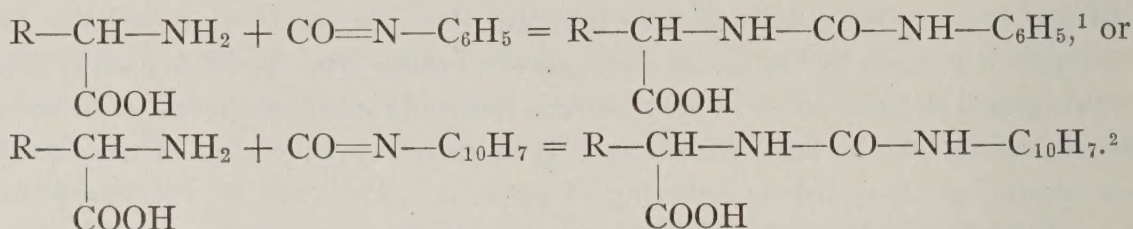
Various characteristic derivatives of amino acids are known. Some of them are very useful in the isolation and identification of amino acids. Among the salts of amino acids, the copper salts are noteworthy because some of them are only slightly soluble in water and, contrary to expectations, they do not form a precipitate of copper hydroxide after treatment with alkalis. The amino acids are best separated by fractional distillation of their ethyl esters. The following derivatives are those used most in the separation and identification of the single amino acids:

1. *Carbamino acids*:

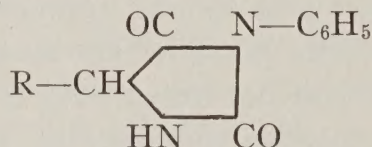


¹ Siegfried. Zeitschr. f. physiol. Chem. 44: 85. 1905; 46: 406. 1905; Siegfried und Neumann. *Ibid.* 54: 423. 1908.

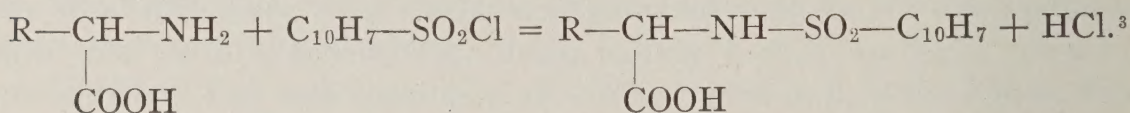
2. *Compounds with phenyl isocyanate or naphthyl isocyanate* (phenyl or naphthyl ureido acids):



On heating with acids these substances split off water and form rings so that the results are crystallized hydantoins:



3. *β-Naphthalene sulpho derivatives*:



All these compounds, like other derivatives, are formed through the high mobility of the amino hydrogen. It will be explained later that this capacity of the amino group for reaction also accounts for the linkage of the amino acids in the protein molecule.

The single proteins do not usually contain all the amino acids that have been isolated. Only one amino acid—arginine—has been isolated from all the proteins so far studied. Both the other diamino acids—histidine and lysine—are also very common and likewise the monoamino acids leucine, tyrosine and glutamic acid. The quantitative distribution of the different amino acids in the protein molecule is also very uneven. Glutamic acid generally predominates in plant proteins and leucine and tyrosine are usually present in considerable quantities.

17. THE INDIVIDUAL AMINO ACIDS

Glycocoll or **glycine** (amino-acetic acid) $\text{CH}_2(\text{NH}_2)-\text{COOH}^4$ is quite soluble in water; m.p. 240° . The copper salt $(\text{NH}_2\text{CH}_2-\text{COO})_2-\text{Cu}$ is rather insoluble. β -Sulpho-naphthalene glycine $\text{COOH}-\text{CH}_2-\text{NH}-\text{SO}_2-\text{C}_{10}\text{H}_7$ has the m.p. 159° .⁵

d-Alanine (α -amino-propionic acid) $\text{CH}_3-\text{CH}(\text{NH}_2)-\text{COOH}^6$ has the m.p. 297° . Very common amino acid. β -Sulpho-naphthalene alanine $\text{COOH}-\text{CH}(\text{CH}_3)-\text{NH}-\text{SO}_2-\text{C}_{10}\text{H}_7$ has the m.p. 123° .⁷

¹ Paal. Ber. chem. Ges. 27: 975. 1894; Paal und Zitelmann. Ibid. 36: 3337. 1903.

² Neuberg und Manasse. Ber. chem. Ges. 38: 2359. 1905; Neuberg und Rosenberg. Biochem. Zeitschr. 5: 456. 1907.

³ Fischer, E. und Bergell. Ber. chem. Ges. 35: 3779. 1902.

⁴ Braconnot, H. Ann. de. chim. et de physique (2) 13: 119. 1820.

⁵ Fischer, E. und Bergell. loc. cit.

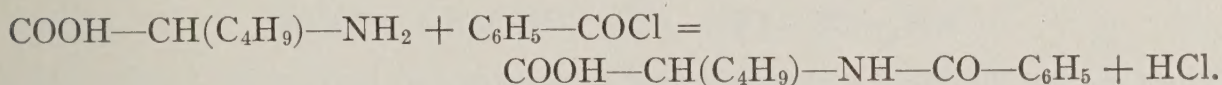
⁶ Schützenberger et Bourgeois. Compt. rend. 81: 1191. 1875; Strecker. Liebig's Ann. d. Chem. 75: 29. 1850 (Synthesis).

⁷ Fischer, E. und Bergell. loc. cit.

1-Serine (α -amino- β -hydroxy-propionic acid) $\text{CH}_2\text{OH}-\text{CH}(\text{NH}_2)-\text{COOH}$.¹ Crystalline flakes; m.p. 293° . Para-nitro-benzoyl serine $\text{COOH}-\text{CH}(\text{CH}_2\text{OH})-\text{NH}-\text{CO}-\text{C}_6\text{H}_4(\text{NO}_2)$ ² has m.p. 190° .

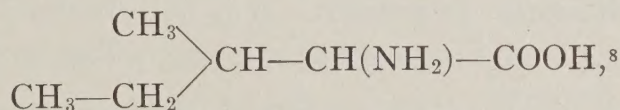
d-Valine (α -amino-isovalerianic acid) $\begin{array}{c} \text{CH}_3 \\ \diagup \\ \text{CH} \\ \diagdown \\ \text{CH}_3 \end{array} - \text{CH}(\text{NH}_2) - \text{COOH}$ ³ has the m.p. 298° . Occurs in small amounts and is hard to separate from leucine. Copper salt is blue-violet. Phenyl isocyanate compound $\text{COOH}-\text{CH}(\text{C}_3\text{H}_7)-\text{NH}-\text{CO}-\text{NH}-\text{C}_6\text{H}_5$ has the m.p. 147° .⁴

1-Leucine (α -amino-isocaproic acid) $\begin{array}{c} \text{CH}_3 \\ \diagup \\ \text{CH} \\ \diagdown \\ \text{CH}_3 \end{array} - \text{CH}_2 - \text{CH}(\text{NH}_2) - \text{COOH}$ has been known the longest⁵ but E. Schulze⁶ was the first to prove the constitution of this important amino acid. Its m.p. is 295° . Soluble in cold water with difficulty. Crystallizes in mother-of-pearl-like flakes. Leucine is a very widespread amino acid which is lacking only in the basic proteins. Its bright-blue copper salt is very characteristic. It is best identified by continued heating with barium hydroxide in the autoclave until it becomes racemic; then it is changed to the benzoyl derivative:



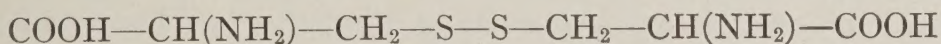
Benzoyl leucine melts at $135-139^\circ$.⁷

d-Isoleucine (methyl-ethyl- α -amino-propionic acid) has the m.p. 280° .



It is more easily soluble in water than l-leucine and occurs only in small amounts. The naphthyl isocyanate compound has the m.p. 178° .⁹

1-Cystine (α -diamino-dithio-dilactyl acid)



is the only protein structural unit containing sulphur that has been isolated so

¹ Cramer, E. Jour. f. prakt. Chem. 96: 76. 1865; Fischer, E. Ber. chem. Ges. 40: 1501. 1907.

² Fischer, E. und W. A. Jacobs. Ber. chem. Ges. 39: 2942. 1906.

³ Gorup-Besanez, E. v. Liebig's Ann. d. Chem. 98: 1. 1856; Schulze, E. Zeitschr. f. physiol. Chem. 22: 417, 423, 425, 427 and 430. 1897; 30: 246, 279 and 289. 1900; and others (Constitution).

⁴ Fischer, E. Ber. chem. Ges. 39: 2320. 1906.

⁵ Proust. Ann. de chim. et de physique (2) 10: 40. 1818; Braconnot. Ibid. 13: 119. 1820.

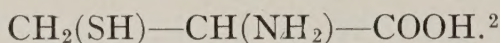
⁶ Schulze, E. und Likiernik. Ber. chem. Ges. 24: 669. 1891; Idem. Zeitschr. f. physiol. Chem. 17: 513. 1893.

⁷ Lippich. Ber. chem. Ges. 39: 2953. 1906.

⁸ Ehrlich, F. Ber. chem. Ges. 37: 1809. 1904; 41: 1453. 1908.

⁹ Neuberg, C. und Manasse. loc. cit.

far.¹ It decomposes easily into two molecules of the secondary (not found in true proteins) amino acid **cystein** (α -amino-thio-lactic acid)

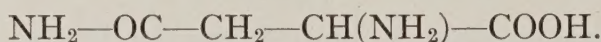


Cystine is soluble in water and in alcohol with difficulty; on boiling with lead salts in alkaline solution it yields the black precipitate of lead sulphide.

l-Aspartic acid (amino-succinic acid)

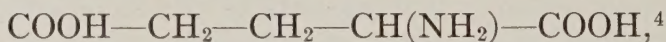


has acid properties and is not very soluble in water. Its m.p. is 270° . In the protein molecule aspartic acid probably occurs in the form of its half amide, asparagine

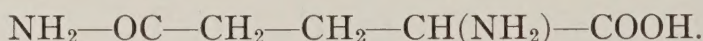


The copper salt of aspartic acid is rather insoluble.

d-Glutamic acid (α -amino-glutaric acid)

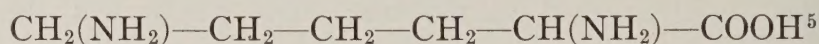


has the m.p. 206° . It occurs in proteins in large quantities. Yeast protein is especially rich in glutamic acid. Has acid properties. In the protein molecule it probably occurs as its half amide, glutamine



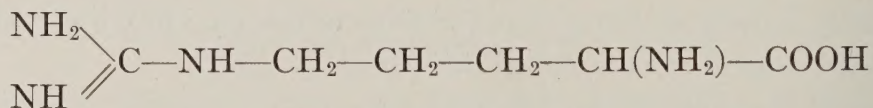
The chlorine hydrate of glutamic acid is insoluble in hydrochloric acid. The m.p. is 193° .

d-Lysine (α , ϵ -diamino-caproic acid)



is a diamino acid with basic properties. It is not obtained in pure crystalline form but yields a splendid crystalline picrate. The m.p. is 252° .⁶ Very common in various proteins.

d-Arginine (guanidine- α -amino-valerianic acid)



is the most essential amino acid because it occurs in all proteins. Some protamines are composed almost exclusively of it but they still have typical protein characteristics.⁷

¹ Wollaston. Phil. Trans. Roy. Soc. London, p. 220. 1810; Mörner, K. A. H. Zeitschr. f. physiol. Chem. 28: 595. 1899; 34: 207. 1901; Friedmann, E. Hofmeister's Beitr. z. chem. Physiol. und Path. 3: 1. 1902.

² Baumann, E. Zeitschr. f. physiol. Chem. 8: 299. 1884; Mörner, K. A. H. Ibid. 34: 287. 1902; Patten, A. J. Ibid. 39: 350. 1903; According to C. Mörner, there must be other sulphur compounds in the protein molecule. Cf. Mörner, C. Ibid. 93: 175. 1914.

³ Ritthausen. Jour. f. prakt. Chem. (1) 107: 218. 1869; Kreusler. Ibid. 107: 240. 1869.

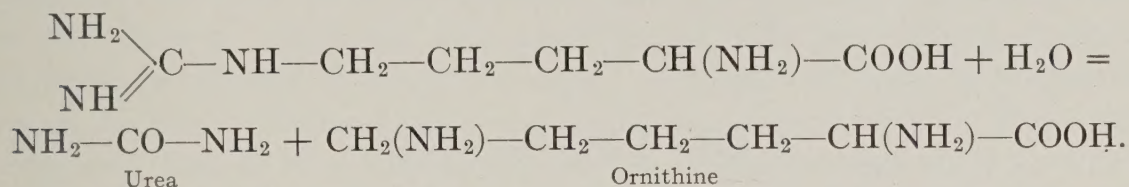
⁴ Ritthausen. Jour. f. prakt. Chem. (1) 99: 454. 1866; 103: 233 and 239. 1868; 106: 445. 1869; 107: 218. 1869.

⁵ Drechsel, E. und R. Krüger. Ber. chem. Ges. 25: 2454. 1892; Drechsel. Ibid. 28: 3189. 1895.

⁶ Kossel, A. und F. Kutscher. Zeitschr. f. physiol. Chem. 31: 165. 1900.

⁷ Kossel, A. und H. Pringle. Zeitschr. f. physiol. Chem. 49: 302. 1906; Kossel, A. Ibid. 44: 347. 1905; and others.

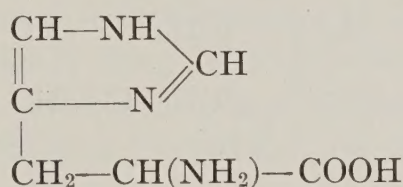
Arginine was first discovered by E. Schulze¹ and Schulze and Winterstein² proved its constitution by synthesis from cyanamide and ornithine. Arginine crystallizes in druses, has the m.p. 207° and is very soluble in water. It has well defined basic properties. It is usually isolated as the basic copper nitrate $(C_6H_{14}N_4O_2)_2Cu(NO_3)_2 + 3H_2O$ with the m.p. 232°. In living tissues of plants there is the enzyme arginase which splits arginine into urea and the secondary amino acid ornithine:³



Arginase had been found previously in animal tissues.⁴

d-Ornithine (α , δ -diamino-valerianic acid)⁵ (*cf.* formula above) has basic properties, is very soluble in water and does not form crystals. β -Naphthalene-sulpho-ornithine has the m.p. 189°.

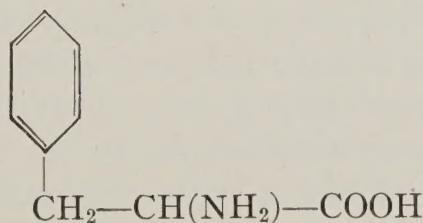
1-Histidine (β -imidazo- α -amino-propionic acid)⁶



is present in proteins in small amounts but is very common. It has basic properties and crystallizes in flakes. The m.p. is 253° . Histidine hydrochloride $\text{C}_6\text{H}_9\text{O}_2\text{N}_3\text{—HCl} + \text{H}_2\text{O}$ crystallizes out in thick colorless prisms with m.p. $251\text{--}252^{\circ}$.⁷

Lysine, arginine and histidine are three basic amino acids which are very important in the building of the protein molecule.

1-Phenylalanine (phenyl- α -amino-propionic acid)⁸



¹ Schulze, E. und E. Steiger. Ber. chem. Ges. 19: 1177. 1886; Idem. Zeitschr. f. physiol. Chem. 11: 43. 1887.

² Schulze, E. und E. Winterstein. Ber. chem. Ges. 30: 2879. 1898; Idem. Zeitschr. f. physiol. Chem. 28: 1. 1898.

³ Kiesel, A. Arginine and its transformations in plants. 1916 (Russian).

⁴ Kossel, A. und Dakin. Zeitschr. f. physiol. Chem. 41: 321. 1904; 42: 181. 1904.

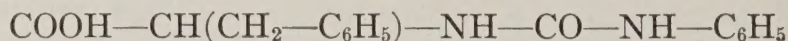
⁵ **Riesser.** Zeitschr. f. physiol. Chem. 49: 238. 1906; **Weiss.** *Ibid.* 59: 499. 1909.

⁶ Discovery: **Kossel, A.** Zeitschr. f. physiol. Chem. 22: 177. 1896; Constitution: **Knoop, F.** und **A. Windaus.** Hofmeister's Beitr. z. chem. Physiol. und Path. 7: 144. 1905; 8: 407. 1906.

⁷ Kossel, A. und F. Kutscher. Zeitschr. f. physiol. Chem. 28: 382. 1899.

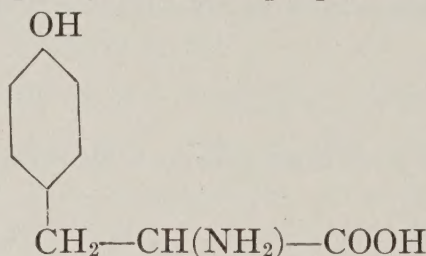
⁸ Schulze, E. und J. Barbieri. Ber. chem. Ges. 14: 1785. 1881.

is slightly soluble in cold water and has the m.p. 283° . The phenyl isocyanate compound of phenylalanine



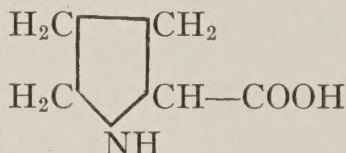
melts at 182° .¹

1-Tyrosine (ρ -hydroxy-phenyl- α -amino-propionic acid)²



is one of the most widespread amino acids. It is very slightly soluble in water (1 part in 2454 parts of water at 20°) and is therefore easily separated from the other amino acids by simple crystallization. It gives a positive Millon's test. Needle-shaped crystals with m.p. $314-318^{\circ}$. All tyrosine derivatives crystallize well.

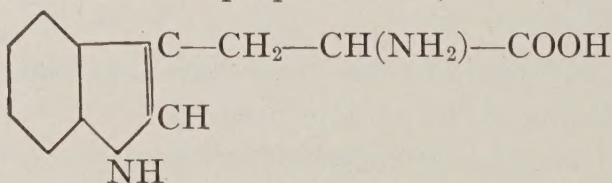
1-Proline (α -pyrrolidine carboxylic acid)³



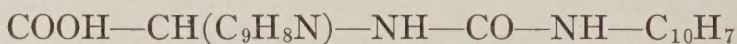
is very soluble in water.

1-Oxyproline (oxy- α -pyrrolidine carboxylic acid)⁴ contains three atoms of oxygen. Forms little tablets with m.p. 270° .

1-Tryptophane (indol- α -amino-propionic acid)⁵



gives some beautiful color tests, some of which have already been mentioned.⁶ Tryptophane is probably the parent substance of some alkaloids. It forms little tablets scarcely soluble in water; m.p. is 289° . The copper salt is hardly soluble in water.⁷ The naphthyl isocyanate compound



melts at 159° .⁸

¹ Fischer, E. und A. Mouneyrat. *Ibid.* 33: 2383. 1900.

² Liebig, J. Liebig's Ann. d. Chem. 57: 127. 1846.

³ Fischer, E. Zeitschr. f. physiol. Chem. 33: 151. 1901.

⁴ Fischer, E. Ber. chem. Ges. 35: 2260. 1902.

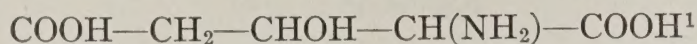
⁵ Hopkins, F. G. and S. W. Cole. Jour. of Physiol. 27: 418. 1901; 29: 451. 1903; Ellinger, A. Ber. chem. Ges. 37: 1801. 1904; Ellinger, A. und C. Flamand. *Ibid.* 40: 3029. 1902.

⁶ Tryptophane yields the "protein tests" of Adamkiewicz and Neubauer (see above) and also the following test. The addition of bromine water to an aqueous solution of tryptophane gives a reddish-violet color. This test was once used to detect a digestion of protein by trypsin which had gone as far as the formation of crystalline products; hence the name tryptophane ($\varphi\alpha\iota\nu\omega$ = evident, manifest).

⁷ Abderhalden, E. und M. Kempe. Zeitschr. f. physiol. Chem. 52: 207. 1907.

⁸ Neuberg, C. und Manasse. Ber. chem. Ges. 38: 2359. 1905; Ellinger, A. und C. Flamand. *loc. cit.*

In addition to these amino acids, **hydroxy-glutamic acid**



and the aliphatic **diamino-oxy-dodecanic acid** $\text{C}_{12}\text{H}_{26}\text{N}_2\text{O}_5$ ² of uncertain constitution have also been found from the hydrolysis of protein. It is quite possible that some other nitrogenous products still remain unknown but important new discoveries in this field are hardly to be expected. We are rather entitled to claim that the most important structural units of the protein molecule are now known. Ammonia and glucosamine have been found in protein hydrolysis in addition to the amino acids. Ammonia is probably a saponification product of the amino groups in the protein molecule. As for glucosamine, it does not seem to be a cleavage product of simple proteins but a part of the carbohydrate complex of the so-called glucoproteids.

E. Fischer and his co-workers³ have synthesized many of the amino acids. In this connection, Fischer's method of synthesis of optically active amino acids is particularly important. Moreover, Fischer has found new methods of separation and isolation of the amino acids. These will be treated later.

If the quantitative proportions of the amino acids obtained from the hydrolysis of proteins are compared with the empirical composition of the proteins that were hydrolyzed, there is always an excess of nitrogen and carbon and a deficit of oxygen in the cleavage products. This shows that substances of unknown nature, but containing oxygen and no nitrogen, are lost in protein hydrolysis by the usual methods.

18. THE CLEAVAGE PRODUCTS OF THE PROTEIDS

It has already been pointed out that proteids are divided into glucoproteids and nucleoproteids. **Glucoproteids** or **mucins** are compounds of proteins with large carbohydrate complexes of undetermined nature. This carbohydrate group probably comes for the most part from a nitrogenous polysaccharide which yields glucosamine on hydrolysis. There is generally a large output of glucosamine in the hydrolysis of glucoproteids.

Nucleoproteids make up most of the substance of nuclei. Gastric juice splits protein-like substances from nucleoproteids and the residues are the so-called **nucleins** which give proteins and **nucleic acids** on further hydrolysis. The protein contained in the nucleoproteids is often a histone or protamine and therefore a substance of relatively low molecular weight. As for the nucleic acids, they are compounds which have nothing to do with proteins because they contain phosphoric acid but no amino acids. The most important distinction between the simple proteins and the nucleoproteids is the presence of nucleic acids in the latter. Total hydrolysis of the nucleic acids gives phosphoric acid, a large carbohydrate group and certain purine and pyrimidine derivatives of weakly basic nature.⁴

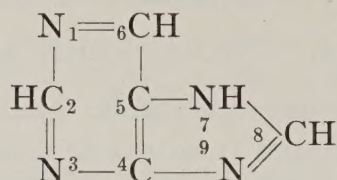
¹ Dakin, H. D. *Biochem. Jour.* 12: 290. 1918.

² Fischer, E. und E. Abderhalden. *Zeitschr. f. physiol. Chem.* 42: 540. 901.

³ Fischer, E. *Aminosäuren, Polypeptide und Proteine.* 1906.

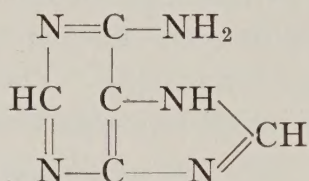
⁴ Kossel, A. *Zeitschr. f. physiol. Chem.* 4: 290. 1880; 7: 7. 1882; 10: 248. 1886; Kossel und Neumann. *Ber. chem. Ges.* 26: 2753. 1893; 27: 2215. 1894; Idem. *Zeitschr. f. physiol. Chem.* 22: 188. 1896.

Purine

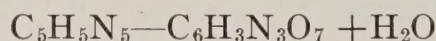


is a substance which does not occur in nature but 2, 6, 8,-trioxypurine is prepared from uric acid by reduction.¹ From the foregoing formula it is evident that the double purine ring is composed of two uric acid residues bound together by a three-carbon chain. Purine is a parent substance of basic compounds which are very important in physiology and are structural units of the nucleic acids. The following purine derivatives are obtained by hydrolysis of the nucleic acids (*cf.* the numbering of the atoms in the purine molecule),

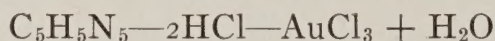
Adenine $\text{C}_5\text{H}_5\text{N}_5 + 3\text{H}_2\text{O}$ (6-amino-purine)²



Crystallizes in long needles like purine itself; scarcely soluble in cold water; m.p. 360–365°; easily oxidized to urea. Adenine picrate

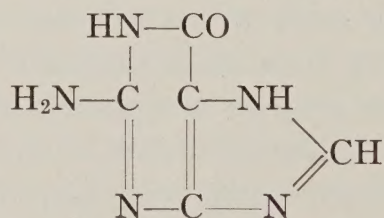


is barely soluble in water at 15–20° but is very soluble in alcohol. It decomposes at 279–281°. The gold chloride adenine salt



decomposes at 215–216°.

Guanine $\text{C}_5\text{H}_5\text{N}_5\text{O}$ (2-amino, 6-oxy-purine)³

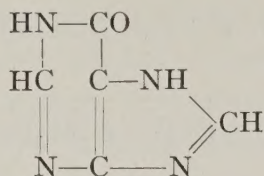


Barely soluble in water, alcohol and ether but soluble in dilute alkalies and mineral acids. Guanine sulphate $\text{C}_5\text{H}_5\text{N}_5\text{O}-2\text{H}_2\text{SO}_4 + 2\text{H}_2\text{O}$ vanishes in water at 120°.

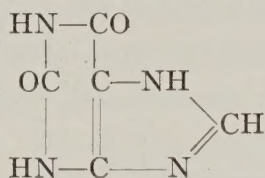
¹ Fischer, E. Synthesen in der Purin- und Zuckergruppe. 1903; Isay, O. Ber. chem. Ges. 39: 250. 1906.

² Kossel, A. Ber. chem. Ges. 18: 78 and 1923. 1885; Idem. Zeitschr. f. physiol. Chem. 10: 250. 1886; 12: 241. 1888; Fischer, E. Ber. chem. Ges. 30: 2226. 1897 (Constitution).

³ Unger. Liebig's Ann. d. Chem. 51: 395. 1844; 58: 18. 1846; Kossel, A. Zeitschr. f. physiol. Chem. 7: 7. 1882; Fischer, E. loc. cit. (Constitution).

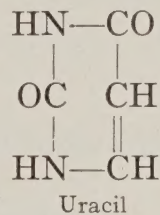
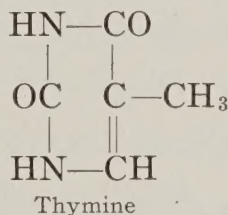
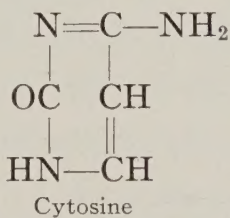
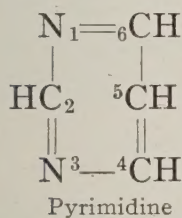
Hypoxanthine $C_5H_4N_4O$ (6-oxy-purine)¹

Microscopic needles; slightly soluble in cold water; thrown out of solution by lead acetate and ammonia. Hypoxanthine nitrate $C_5H_4N_4O \cdot HNO_3 + H_2O$ is especially characteristic. Hypoxanthine is an oxidation product of adenine. According to modern ideas,² there is no hypoxanthine in true nucleic acids.

Xanthine $C_5H_4N_4O_2$ (2, 6-oxy-purine)³

Barely soluble in water; decomposes on heating (like guanine and hypoxanthine). Xanthine nitrate forms spherical dense crystals. Xanthine is also a secondary product which comes from the oxidation of guanine and is not contained in the molecule of nucleic acid.

The parent substance of pyrimidine bases is pyrimidine, a synthetic substance which melts at 22° and does not occur in nature.

Cytosine (2-oxy, 6-amino-pyrimidine)⁴

Little flakes which glitter like mother-of-pearl. Does not melt but decomposes at $320-325^\circ$. Barely soluble in water, alcohol and ether. Cytosine picrate $C_4H_5N_3O \cdot C_6H_3N_3O_7$ has the m.p. 270° .

Thymine (5-methyl, 2, 6-dioxy-pyrimidine).⁵ Crystals have an oily sheen. Decomposes at 321° . Soluble in hot water, slightly soluble in cold water, alcohol and ether.

Uracil (2, 6-dioxy-pyrimidine)⁶ is very soluble in hot water and slightly soluble in cold water. Decomposes at 338° . Does not form a picrate. According to some investigators, uracil is a secondary product. From the foregoing explanation it is evident that only the presence of four bases—adenine, guanine, cytosine and thymine—is undoubtedly proved. The total of the nitrogenous

¹ Scherer. Liebigs Ann. d. Chem. 73: 328. 1850; Fischer, E. Ber. chem. Ges. 30: 2226. 1897 (Constitution).

² Schmiedeberg. Arch. f. exp. Path. und Phar. 43: 57. 1900; Steudel. Zeitschr. f. physiol. Chem. 43: 402. 1904; 53: 14. 1907; Kowalewski. Ibid. 69: 248. 1910; and others.

³ Liebig und Wöhler. Liebigs Ann. d. Chem. 26: 340. 1838; Fischer, E. loc. cit. (Constitution).

⁴ Kossel, A. und H. Steudel. Zeitschr. f. physiol. Chem. 37: 177 and 377. 1903; 38: 49. 1903 (Constitution).

⁵ Kossel, A. und A. Neumann. Ber. chem. Ges. 27: 2215. 1894; Steudel, H. Zeitschr. f. physiol. Chem. 32: 241. 1901 (Constitution).

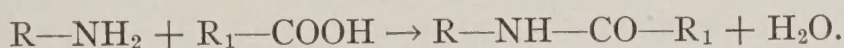
⁶ Ascoli. Zeitschr. f. physiol. Chem. 31: 161. 1900; Steudel, H. loc. cit. (Constitution.)

Among the plant nucleic acids, those of wheat seedlings¹ and yeast² are probably identical. The constitution of the nucleic acid of yeast has not been determined.³ The most probable empirical formula for it is $C_{38}H_{49}N_{15}O_{29}P_4$.⁴ By cautious splitting of yeast nucleic acid, mononucleotids have been recently isolated as pure brucine salts.⁵ All these new achievements lead us to hope that we shall not have to wait much longer for the complete exposition of the constitution of nucleic acids, the most important chemical constituents of cell nuclei.

Pure preparations of nucleic acids are amorphous, highly hygroscopic powders which readily dissolve in alkalis to form salts. These salts are easily broken down by warming with acids. The capacity of nucleic acids to remove protein from solution is especially interesting.⁶ The resulting precipitates are surprisingly similar to the natural nucleins.

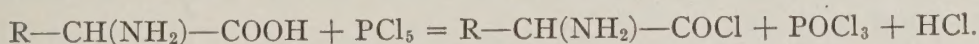
19. POLYPEPTIDES

After the importance of the amino acids in the protein molecule had been proved beyond doubt, there arose the question whether the amino acids could be put together so that the product would resemble the proteins or at least the large cleavage products (see below). This problem was solved in brilliant fashion by E. Fischer.⁷ He called the resultant products polypeptides by analogy with polysaccharides. Just as in the polysaccharides, we have the groups di-, tri-, tetrapeptides, etc. In polypeptides there is an amide-like union between the amino group of one amino acid and the carboxyl group of another amino acid:

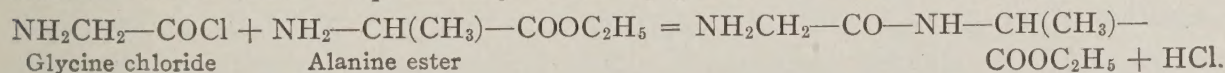


Fischer proposed various methods to bring about these unions.

The most general and the most important of these methods was as follows. One amino acid was changed to a chloride by chlorination (with PCl_5 in acetyl chloride):



The other amino acid was changed to the ethyl ester (*cf.* analytical methods [p. 286]). The reaction between these two products gives the dipeptide ester which is easily saponified



¹ Osborne, T. B. and J. F. Harris. Amer. Jour. Physiol. 9: 69. 1903; Levene und La Forge. Ber. chem. Ges. 43: 3164. 1910.

² Slade. Amer. Jour. Physiol. 13: 464. 1905; Boos, W. F. Jour. Biol. Chem. 5: 469. 1909; Kowalewsky, K. Zeitschr. f. physiol. Chem. 69: 248. 1910; Levene und Jacobs. Ber. chem. Ges. 42: 1198, 2469, 2474 and 2703. 1909; 43: 3150 and 3162. 1910; 44: 740 and 1027. 1911; 45: 608. 1912; and others.

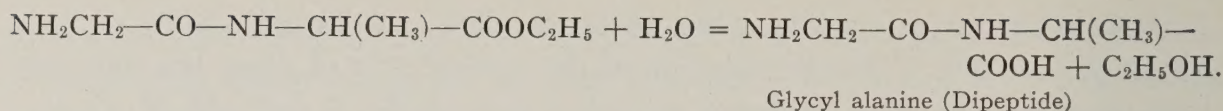
³ Kowalewsky, K. *loc. cit.*; Steudel, H. Zeitschr. f. physiol. Chem. 108: 42. 1919; Levene und Jacobs. *loc. cit.*

⁴ Levene und Jacobs. *loc. cit.*

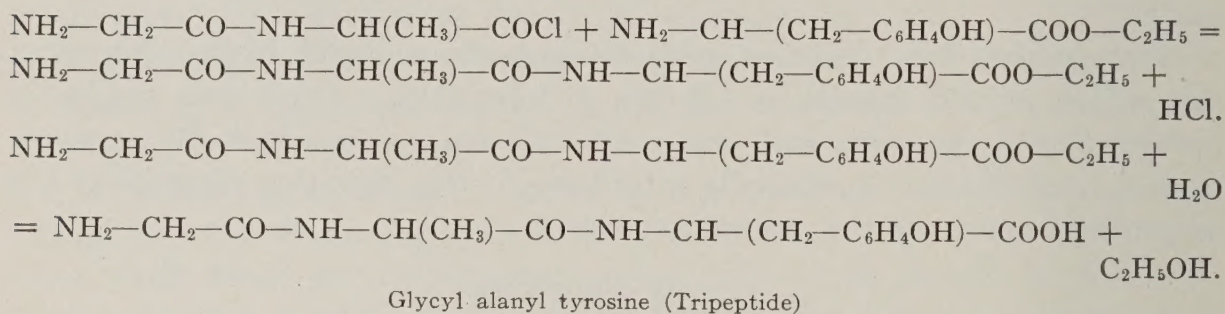
⁵ Levene. Jour. Biol. Chem. 31: 591. 1918; 33: 229 and 425. 1918; Thannhauser und Dorfmueller. Zeitschr. f. physiol. Chem. 95: 259. 1915; 100: 121. 1917; 104: 65. 1919; 107: 157. 1919; 109: 177. 1920.

⁶ Altmann, K. Arch. f. Anat. und Physiol. for 1889, p. 524; Milroy, T. Zeitschr. f. physiol. Chem. 22: 307. 1896.

⁷ Fischer, E. Untersuchungen über Aminosäuren, Polypeptide und Proteine. 1906.

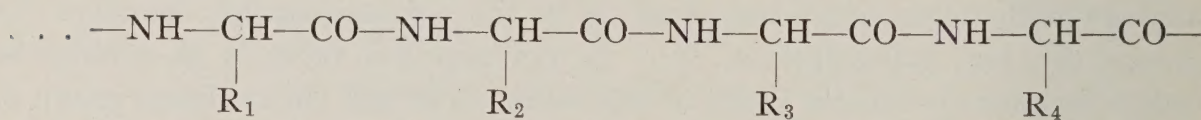


Isomeric alanyl glycine is formed when the alanine is chlorinated and the ester made from the glycine. Since the polypeptides can be chlorinated without decomposition it is possible to lengthen both ends of the dipeptide chain. For example, tyrosine can be added to glycyl alanine in the following manner:



It is evident that two peptides can be thus united and large molecular complexes obtained quickly. The largest polypeptide that has been set together¹ has a molecular weight of 1326 and consists of 19 amino acids.

Thus the complicated polypeptides are chains of the following structure:



etc.

This chain is a repetition of the grouping $-\text{NH}-\text{CH}-\text{CO}-$ and the other parts of the individual amino acids are represented as side chains $\text{R}_1, \text{R}_2, \text{R}_3, \text{R}_4$, etc. The configuration of the polypeptide requires that the NH_2 group be in the alpha position in all amino acids and this is true. On hydrolysis the long chains break at the points of least resistance which are between CO and NH, and the amino acids are set free.

There are good reasons for thinking that there are polypeptide unions in true proteins. In the first place, incomplete hydrolysis of proteins gives polypeptides which are identical with synthetic products.² Secondly, it has been shown that the specific enzymes of plant and animal cells split polypeptides to amino acids.³ This is very important in view of the specificity of proteolytic enzymes. It has been observed that these enzymes are unable to break up synthetic polypeptides in which a single amino acid does not have the sign of rotation characteristic of the natural products.⁴ This is evident from the following table:

¹ Abderhalden, E. und Fodor. Ber. Chem. Ges. 49: 561. 1916.

² Fischer, E. und E. Abderhalden. Ber. chem. Ges. 39: 752 and 2315. 1906; 40: 3544. 1907; and others.

³ Abderhalden, E. et al. Zeitschr. f. physiol. Chem. 47: 359 and 466. 1906; 48: 537. 1906; 49: 1, 21 and 31. 1906; 51: 334. 1907; 53: 280, 294 and 308. 1907; 55: 371, 377, 390 and 395. 1908; and others.

⁴ Fischer, E. und E. Abderhalden. Zeitschr. f. physiol. Chem. 46: 52. 1905; 51: 264. 1907.

Can be hydrolyzed	Cannot be hydrolyzed
d-Alanyl-d-alanine	d-Alanyl-l-alanine
d-Alanyl-l-leucine	l-Alanyl-d-alanine
l-Leucyl-l-leucine	d-Leucyl-l-leucine
l-Leucyl-d-glutamic acid	d-Leucyl-l-leucine

Thus the enzymes break down only the polypeptides which occur in living cells.

It is noteworthy that there is a gradual change in the properties of the series of synthetic polypeptides as the molecular weight increases. Different facts in the realm of astronomy indicate that the mass of a body has a *qualitative* influence on many of its properties. The properties of the whole are not merely the sum of those of its parts. Mere increase in mass may introduce entirely new properties. For example, a planet can support higher forms of life only when its mass is large enough to give it an atmosphere by gravitational attraction. Thus there is a "critical point" below which many phenomena are impossible. All planets with a mass below this critical point differ from the larger planets by the non-existence of higher life on their surfaces. For the same reason a small sun would be impossible because it requires considerable size to allow for enough shrinkage to produce heat enough to be luminous.¹

In like manner, there is a transition from amino acids to proteins which at first glance seem entirely different. Polypeptides are the transition stages. Di- and tripeptides of low molecular weight have in general the same properties as the amino acids of which they are formed and are therefore crystalline and yield typical copper salts and derivatives of β -naphthalene and phenyl- and naphthyl-isocyanate. Larger polypeptides have properties unlike those of the amino acids. For example, even polypeptides are amorphous substances which have no sharp melting point and often give the biuret test which is negative with amino acids and the more simple polypeptides. Polypeptides of still higher molecular weight again show new properties which are much like those of proteins: their solutions are opalescent and, like protein solutions, can be precipitated by phospho-tungstic acid and by tannic acid. The solutions of large polypeptides form foams even when much diluted. Thus it is evident that they resemble simple proteins more than amino acids.

20. PROTEIN CLEAVAGE BY ENZYMES

It has long been known that proteins in the stomach are split by the enzyme *pepsin* with the formation of the so-called *peptones*.² Peptones are amorphous substances which give all reactions for proteins including the important biuret

¹ Lodge, O. Life and Matter. 1905.

² Payen. Compt. rend. 17: 654. 1843.

test. Unlike typical proteins, peptones are not salted out, do not coagulate when boiled and they diffuse through some membranes. The so-called albumoses were once separated from the peptones because they could be salted out and thus according to the prevailing ideas, were said to have a higher molecular weight. Investigations of polypeptides have shown, however, that the capacity to be thrown out of solution under the influence of various salts depends not on the size but on the qualitative composition of the molecule.¹ For example, polypeptides containing tyrosine are easily salted out even when the molecular weight is relatively low. This removes the only distinction between albumoses and peptones and the term albumose has become superfluous.²

In the small intestine the pancreatic juice produces a complete hydrolysis of proteins to crystalline amino acids. The enzyme which brings about this cleavage was discovered by Claude Bernard³ and named *trypsin*. The view has repeatedly been expressed that trypsin is not an entity but consists of two enzymes; one is pepsin, or according to the rational nomenclature—*protease*, which changes protein to peptones and the second is *erepsin* or *peptase* which hydrolyzes peptones to amino acids.⁴ Other workers consider that trypsin is a single enzyme and the solution of the controversy depends on future investigations. In any case it must be borne in mind that there is no doubt of the existence of erepsin as an enzyme which splits only peptones and has no effect on proteins.⁵

There are analogous enzymes in plants. It has long been known that the latex of the pawpaw tree (*Carica Papaya*) produces a hydrolytic cleavage of proteins.⁶ The enzyme which was isolated from this juice was named papayotin.⁷ Its effect is identical with that of trypsin. Proteolytic enzymes were later found in various plants. Germinating seeds are especially rich in them.⁸ Powerful proteolytic enzymes have been detected in yeast.⁹ In seedlings of seed-plants and in yeast cells, the action of the proteolytic enzymes is analogous to that of trypsin. Still it is thought that the hydrolysis of protein in plants is also divided into two stages; some plant enzymes are like animal pepsin and others are analogous to erepsin or peptase.

¹ Fischer, E. und E. Abderhalden. Ber. chem. Ges. 40: 3544. 1907; Fischer, E. Ibid. 40: 3704. 1907.

² Abderhalden, E. Neuere Ergebnisse auf dem Gebiete der spez. Eiweisschemie, p. 14. 1909.

³ Bernard, Cl. Lecons de physiol. exp., p. 334. 1855.

⁴ Pollak, L. Hofmeister's Beitr. z. chem. Physiol. und Path. 6: 95. 1904; Mays, K. Zeitschr. f. physiol. Chem. 49: 124 and 188. 1906; Marras, Fr. Centralbl. f. Bakt. (I) 75: 193. 1914.

⁵ Cohnheim, O. Zeitschr. f. physiol. Chem. 33: 451. 1901; 35: 134. 1902; 36: 13. 1902; 47: 286. 1906; 49: 64. 1906; 52: 526. 1907; Salaskin, S. Ibid. 35: 419. 1902; Vernon, H. M. Jour. of Physiol. 32: 33. 1905; 33: 81. 1905; and many others.

⁶ Vauquelin. Ann. de chim. 43: 267. 1802.

⁷ Wurtz, A. et E. Bouchut. Compt. rend. 90: 1379. 1880; 91: 787. 1880.

⁸ Green, J. R. Phil. Trans. Roy. Soc. London (B) 177: 39. 1887; Idem. Proc. Roy. Soc. London. 41: 466. 1886; 47: 146. 1890; 48: 370. 1891; Butkewitsch, W. Protein Decomposition in the Seed-plants and the Rôle of the Proteolytic Enzymes. 1904 (Russian).

⁹ Geret und Hahn. Ber. chem. Ges. 31: 202 and 2335. 1898; cf. also "Zymasegärung" 1904; Salkowski, E. Zeitschr. f. physiol. Chem. 31: 305. 1900; Dernby, K. Biochem. Zeitschr. 81: 107. 1917; Iwanoff, N. N. Investigations of the Transformations of Nitrogenous Substances in Yeast. 1919 (Russian).

A considerable splitting of proteins to peptones was observed first in the insectivorous plants¹ but it was then found that other plants² as well as yeast³ had two kinds of proteolytic enzymes—proteases and peptases.

After polypeptides had been synthesized, the question arose whether the products of protein digestion—albumoses and peptones—are chemically individual substances. It has been pointed out above that the term albumose is now obsolete. Some workers claimed that peptones are nothing but an unstable mixture of polypeptides. On the contrary, others assumed that peptones are definite structural units of the protein molecule, with constant chemical composition and characteristic properties. The remarkable discovery was made long ago that, in protein cleavage by enzymes, complexes rich in tyrosine and tryptophane are the first to appear in solution and then the more stable residue composed of glycocoll, phenylalanine and proline becomes hydrolyzed.⁴ Of course, of itself this fact is not a definite proof of the existence of individual peptones because there are other possible explanations.⁵ Still among the products of partial protein hydrolysis there was found an interesting group of peptones which have a rather constant composition, a proportionally lower molecular weight and marked basic properties. These substances were named **kyrins**.⁶ The cleavage of glucokyrin showed that this substance is composed largely of the diamino acids arginine and lysine. Kossel⁷ assumes that such protamine-like complexes composed of diamino acids form the central nucleus of every protein molecule. According to Kossel, this protamine group is very important in the processes of reproduction, heredity and formation of tissues and organs. This theory states that the basic proteins in fish sperms especially are distinct from the inactive constituents of the ordinary protein molecule, which consist of monoamino acids.

A whole series of investigations of the formation of peptones from proteins make it probable that this is not a hydrolytic cleavage but merely a change of the disperse condition of the colloidal constituents of the protein complex. There is no increase in NH₂ and COOH groups when protein is split by pepsin⁸ but the internal friction [of the solution] is thereby lowered considerably. Peptones may form even at 100°⁹ and at 80–90° a surprisingly large amount of “albumoses” and “peptones” appear *in one minute* in a mixture of protein and papayotin.¹⁰ All these results agree with the work of S. Perow and G. Inichow to be considered later.

There are specific enzymes—the so-called nucleases¹¹—for the cleavage of nucleic acids. The nuclein metabolism of plants has not been sufficiently studied.

¹ Vines, S. H. Jour. Linnean Soc. 15: 427. 1877; Idem. Ann. Bot. 11: 563. 1897; 12: 545. 1898; 15: 563. 1901; Clautriau, G. Mém. publ. par l'acad. Belg. 1900; White. Proc. Roy. Soc. London (B) 83: 134. 1910; Dernby. Biochem. Zeitschr. 78: 197. 1916; and others.

² Vines, S. H. Ann. Bot. 18: 289. 1904; 19: 149 and 171. 1905; 20: 113. 1906; 22: 103. 1908; 23: 1. 1909; 24: 213. 1910; Blagowestschensky, A. Jour. russ. bot. Ges. 4: 52. 1919 (Russian); Dean. Bot. Gaz. 39: 521. 1905; 40: 121. 1905; and others.

³ Vines, S. H. *loc. cit.*; Dernby, K. Biochem. Zeitschr. 81: 107. 1917; Iwanoff, N. N. *loc. cit.*

⁴ Kühne, W. und R. H. Chittenden. Zeitschr. f. Biol. 19: 159. 1883; 22: 423. 1885; Pick. Zeitschr. f. physiol. Chem. 28: 219. 1899; Fischer, E. und E. Abderhalden. *Ibid.* 39: 81. 1903; Siegfried. *Ibid.* 38: 259. 1903.

⁵ Fischer, E. und E. Abderhalden. Zeitschr. f. physiol. Chem. 46: 52. 1905; 51: 264. 1907; and others.

⁶ Siegfried. Zeitschr. f. physiol. Chem. 43: 44. 1904; 48: 54. 1906; 50: 163. 1906; 84: 288. 1913; 97: 233. 1916.

⁷ Kossel, A. Zeitschr. f. physiol. Chem. 22: 176. 1896; 25: 165. 1898; Kossel und F. Kutscher. *Ibid.* 31: 213. 1900; Kossel und H. Dakin. *Ibid.* 41: 407. 1904.

⁸ Spriggs, E. Zeitschr. f. physiol. Chem. 35: 465. 1902.

⁹ Delezenne, C., H. Mouton et E. Pozerski. Compt. rend. soc. de biol. 60: 60 and 309. 1906.

¹⁰ Jonescu, D. Biochem. Zeitschr. 2: 177. 1907.

¹¹ Abderhalden, E. und Schittenhelm. Zeitschr. f. physiol. Chem. 47: 452. 1906; Kikkoji. *Ibid.* 51: 201. 1907; Tsuji, K. *Ibid.* 87: 378. 1913; de la Blanchardière. *Ibid.* 87: 291. 1913; Theodoresco, E. C. Compt. rend. 155: 554. 1912; 156: 1081. 1913.

21. ENZYMATIC SYNTHESSES AND THEORIES OF THE CONSTITUTION OF PROTEINS

In addition to the enzymes described on the preceding pages, there occurs in the stomach the interesting curdling enzyme or chymosin which forms an insoluble curd from the casein of milk.¹ Such enzymes are extraordinarily common in plants. They have been found in various fruits, flowers, in latex, etc.² I. Pawlow³ was the first to develop the idea that there is no independent chymosin but that the protein is coagulated by the pepsin which, like all catalysts, influences the reaction in both directions (*cf.* p. 33 and 40), and therefore condenses as well as splits protein. There has previously appeared a series of papers from the school of A. Danilewsky⁴ on the synthetic action of gastric juice. Various workers have found in concentrated solutions of the products of pepsin digestion, peculiar precipitates which were regarded as the result of enzymatic synthesis of protein.⁵ These precipitates are called *plasteins*. Analogous processes have been observed by some investigators under other conditions.⁶ The objections to the view that enzymatic syntheses have taken place in these cases centered on the point that it was more probable that it was a purely physical process of flocculation. However, in recent times the idea has developed that most proteins are not individual chemical compounds but mixtures of various colloidal constituents, *i.e.*, peptones and the complicated polypeptides.⁷ According to this view, pepsin is said to change the disperse condition of the colloidal complexes in the protein solution and to exert no chemical effect. According to this, pepsin would not be an enzyme and a catalyst because this idea presupposes a chemical action.

The latest investigations of protein cleavage agree with these views. By using methods of protein cleavage which involve heating for an hour or two with 1% hydrochloric, sulphuric or phosphoric acid in the autoclave at 180°, W. Ssadikow and N. Zelinski⁸ obtained slightly colored solutions which were free from melanins and yielded 7–13% of the raw materials as cyclic anhydrides of amino acids (diketopiperazines). Those isolated by these investigators include phenyl-glycyl-glycine anhydride, prolyl-proline anhydride and other analogous substances.

¹ Literature by Fuld. *Ergeb. de Physiol.* 1(I): 472. 1902.

² Javillier, M. *Contributions à l'étude de la présure chez les végétaux.* 1903; and many others.

³ Pawlow, I. und S. Paraschuk. *Zeitschr. f. physiol. Chem.* 42: 415. 1904; *cf.* also Herzog, R. *Ibid.* 60: 306. 1909; Migai und Sawitsch. *Ibid.* 63: 405. 1909; Funk und Niemann. *Ibid.* 68: 263. 1910; Graber. *Jour. Ind. Eng. Chem.* 9: 1125. 1917.

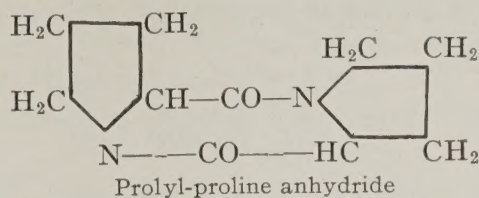
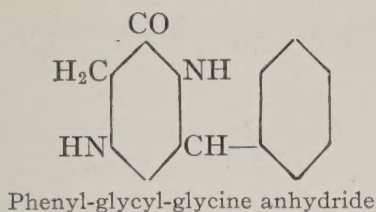
⁴ Okunew. *Diss. Petersburg.* 1895 (Russian); Lawrow. *Diss. Petersburg.* 1897 (Russian); Sawialow. *Pfuger's Arch. f. ges. Physiol.* 85: 171. 1901; Kurajew. *Hofmeister's Beitr. z. chem. Physiol. und Path.* 1: 112. 1901; 4: 476. 1904; Glagolew. *Biochem. Zeitschr.* 50: 162. 1913; 56: 195. 1913; Lawrow. *Zeitschr. f. physiol. Chem.* 51: 1. 1907; 53: 1. 1907; 56: 343. 1908; 60: 520. 1909; Henriques und Gjaldbak. *Ibid.* 71: 485. 1911; 81: 439. 1912; and others; *cf.* p. 41.

⁵ Herzog, R. O. *Zeitschr. f. physiol. Chem.* 39: 305. 1903; Winogradow. *Pfugers Arch. f. ges. Physiol.* 87: 170. 1901; Grossmann. *Hofmeisters Beitr. z. chem. Physiol. und Path.* 6: 192. 1904; 7: 165. 1905; Micheli, F. *Arch. Ital. di Biol.* 46: 185. 1907; and others.

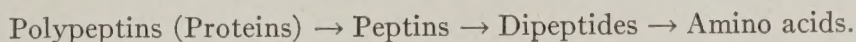
⁶ Robertson, T. B. *Jour. Biol. Chem.* 3: 95. 1907; 5: 493. 1909; Taylor, A. *Ibid.* 3: 87. 1907; 5: 381. 1909; Blagowestschensky, A. *Tagebuch d. 1 Kongr. d. russ. Bot.*, p. 21. 1921 (Russian).

⁷ Peroff, S. *Mitt. d. milchwirtschaft. Inst. in Wologda.* 2: 163. 1921 (Russian); Inichow, G. *Biochem. Zeitschr.* 131: 47. 1922.

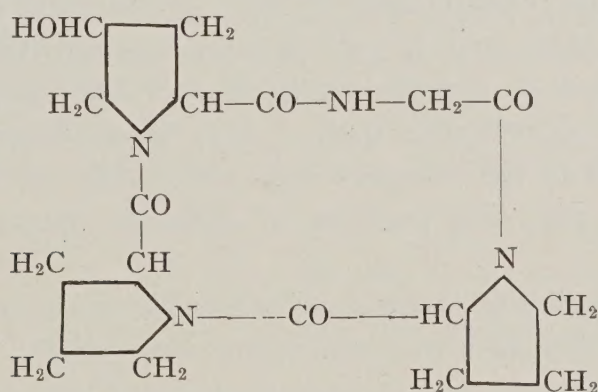
⁸ Ssadikow und Zelinski. *Biochem. Zeitschr.* 136: 241. 1923; 137: 397 and 401. 1923; 147: 30. 1924; Ssadikow. *Ibid.* 143: 496 and 504. 1923; and others.



Ssadilow and Zelinski think that the "protein molecules" are made up of cyclic systems of diketopiperazines, the so-called "peptins." According to them, protein cleavage is said to proceed as follows:



Troensegaard¹ has found another method of splitting proteins. He forms an acetyl of gliadin dissolved in glacial acetic acid and then treats it with metallic sodium in the presence of amyl alcohol. This forms alkaloid-like substances which are predominantly pyrrol derivatives. This method brings about considerable modification in the structural units of protein but it hardly proves that there is a pyrrol ring in the protein molecule. Brigl² seeks to prove that there is a secondary formation of diketopiperazines from amino acids in the experiments of Ssadikow and Zelinski, a condition already known in some other cases.³ Still the thorough investigations by Abderhalden and his co-workers⁴ leave little room for doubt that in the protein molecule there are cyclic complexes which are put together like diketopiperazines and which often form complex systems of rings. Thus these investigators isolated from the hydrolysis of horsehair the following compound composed of two prolines, one oxyproline and one glycoll. This compound was not attacked by proteolytic enzymes.



Abderhalden and his co-workers have also synthesized chains of diketopiperazines and amino acids.^w Among these synthetic products the 1, 4-diglycyl-glycine anhydride is inter-

¹ Troensegaard, N. *Zeitschr. f. physiol. Chem.* **127**: 137. 1923; Troensegaard und J. Schmdt. *Ibid.* **133**: 116. 1924.

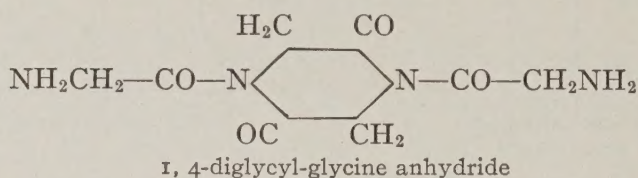
² Brigl. *Ber. chem. Ges.* **56**: 1887. 1923.

³ Fischer, E. und E. Abderhalden. *Ber. chem. Ges.* **39**: 752. 1906; Dakin. *Biochem. Jour.* **12**: 290. 1918; and others.

⁴ Abderhalden, E. und H.. Suzuki. *Zeitschr. f. physiol. Chem.* **127**: 281. 1923; Abderhalden, E. *Ibid.* **128**: 119. 1923; **131**: 280 and 284. 1923; Abderhalden und E. Klarmann. *Ibid.* **129**: 320. 1923; Abderhalden und W. Stix. *Ibid.* **129**: 143. 1923; **132**: 238. 1924; Abderhalden und E. Komm. *Ibid.* **132**: 1. 1924; **134**: 113 and 121. 1924; **136**: 134. 1924; Abderhalden, Klarmann und Schwab. *Ibid.* **135**: 180. 1924; Abderhalden, E. *Naturwissenschaften.* **5**: IX. 1924; [cf. the review by Klarmann. *Chem. Rev.* **4**: 51-107. 1927.]

^w In their criticism of the work of Abderhalden on the constitution of the protein molecule, Osborne and Vickery (*Physiol. Rev.* **8**: 393. 1928) point out that none of the synthetic products of this line of attack are acted on by the proteolytic enzymes.—Ed.

esting. It is a crystalline substance, gives the biuret test and is precipitated by phosphotungstic acid.



On the basis of the foregoing results, it is a plausible hypothesis that proteins are not individual molecules but are composed of complex labile unions of various independent systems of rings. The ring systems are probably formed by polymerization of diketopiperazines. According to this, the first stage in protein cleavage is nothing but a liberation of these systems of rings. This coördinates the apparently inconsistent work on the formation of peptones and plasteins, on the purely physical nature of the action of pepsin and on the cyclic products of protein cleavage. In any case, there is no doubt but that a new epoch of protein investigation is commencing.

In conclusion, it is to be emphasized that the latest studies have shown a certain similarity in the structure of polysaccharides and that of proteins. From the modern standpoint, neither the "polysaccharides" nor the "proteins" are homogeneous molecules but merely express a definite disperse condition of their colloidal constituents (*cf.* p. 243 ff.).

22. THE DIVERSITY OF PROTEINS AND THEIR RATIONAL CHARACTERISTICS

The various modern theories of heredity, immunity, etc. are supported by the fact that every kind of living thing has its specific proteins. Is it actually possible to have such a variety of these substances? Simple calculations of the number of possible permutations for the combinations of amino acids and polypeptides¹ show that 1216×10^{17} different polypeptides can be formed from 19 members; from 30 members, 2653×10^{32} different synthetic products can be prepared. Theoretically, then, a very great diversity of proteins is possible. The fact that the enzymes only act rapidly on the proteins of the parent plant shows that the proteins of different plants are actually not identical.²

A general classification of plant proteins was given above (p. 266), based on solubility and the conditions of their removal from solution. A strictly chemical classification of proteins can only be based on quantitative hydrolysis and the determination of the percentages of the single amino acids in the protein molecules. Such determinations have been carried out in large numbers by Osborne and his associates. From these it is found that many proteins which have been regarded as identical according to the percentages of the single elements and to their properties, are actually different when judged by the amino acids in their molecules. At the same time, Osborne and his school have improved the methods of isolating proteins so that the plant proteins have now been investigated more completely than those from animals. Osborne has obtained the characteristics of every protein by a series of analyses and, although the appropriate papers have not all appeared, thorough descriptions have already been given even of proteins which Osborne's predecessors did not know existed.

¹ Fischer, E. *Zeitschr. f. physiol. Chem.* 99: 54. 1917.

² Blagowestschensky, A. *Tagebuch. d. I Kongr. d. russ. Bot.*, p. 21. 1921 (Russian); and others.

Some facts may be given here of the most typical, widespread and available proteins.

I. Globulins (p. 266). *Legumin*¹ in the seeds of peas, Windsor beans, vetch and lentils. Does not coagulate easily, dissolves in 5-10% NaCl, and is salted out by 46-67% of complete saturation. Nitrogen content 18.00%, including 1.7% ammonia N, 11% monoamino acid N and 5.1% diamino acid N.

*Vicillin*² in the same legumes. Coagulates more easily than the preceding one, dissolved in 1-2% NaCl and is salted out by 70-80% of complete saturation. Nitrogen content 17%, including 1.75% ammonia N, 4.6% diamino acid N and 10.4% monoamino acid N.

*Phaseolin*³ in *Phaseolus vulgaris*. Dissolves in very weak NaCl solutions and is salted out between 57% and 77% of complete saturation. Does not coagulate easily.

Conglutin α^4 together with *conglutin* β in seeds of the yellow lupine. Does not coagulate but the solution sets to a jelly on cooling. Dissolves easily in 10% NaCl. Is salted out by 34-63% of complete saturation with ammonium sulphate. Nitrogen content 17.6%, including 5.2% diamino acid N, 10.5% monoamino acid N and 2.1% ammonia N.

*Edestin*⁵ from hemp (*Cannabis sativa*). Does not coagulate completely, dissolves in neutral salts and is salted out by 23-35% of complete saturation with ammonium sulphate. Does not contain a trace of a carbohydrate group. The total nitrogen amounts to 18.7%.

II. Albumins (p. 266). *Leucosin*⁶ makes up 0.4% of wheat seeds. Coagulates at 50-60°, dissolves in water and is salted out by half saturation with ammonium sulphate. N = 16.8%, including 3.5% diamino acid N and 11.8% monoamino acid nitrogen.

III. Prolamines (p. 266). *Gliadin*⁷ from wheat gluten. Coagulates when boiled and does not dissolve in neutral salts. Contains no trace of phosphorus. N = 17.66%, including 1% diamino acid N, 12.2% monoamino acid N and 4.30% ammonia N.

*Hordein*⁸ from barley seeds. Coagulates only when boiled in water or very dilute alcohol. Does not give the Millon reaction (p. 265). N = 17.2%, including only 0.77% diamino acid N, 12% monoamino acid N and 4% ammonia N.

*Zein*⁹ from maize seeds. Dissolves in 75-90% alcohol. Does not contain glycocoll, lysine or tryptophane and is therefore not an adequate protein food for animals. Like most reserve proteins of plants, it has no carbohydrate group. N = 16.13%, including 0.49% diamino acid N, 12.5% monoamino acid N and 2.97% ammonia N. 80.5% of its weight has been recovered in the products of its hydrolysis (Osborne and Jones).

¹ Osborne and Harris. Jour. Biol. Chem. 3: 213. 1907; Idem. Jour. Amer. Chem. Soc. 25: 32; and 837. 1903; Osborne and Campbell. Ibid. 18: 583. 1896; 20: 342, 362, 393, 406 and 410. 1898; Osborne. Ibid. 24: 39 and 140. 1902; Idem. Zeitschr. f. physiol. Chem. 33: 240. 1901; Idem and Clapp. Jour. Biol. Chem. 3: 219. 1907; Osborne and Heyl. Amer. Jour. Physiol. 22: 423. 1908.

² Osborne and Harris. Jour. Biol. Chem. 3: 213. 1907; Osborne and Heyl. Ibid. 5: 187. 1908; Osborne and Campbell. loc. cit.

³ Osborne and Clapp. Amer. Jour. Physiol. 18: 295. 1907; Osborne. Jour. Amer. Chem. Soc. 16: 633, 703 and 757. 1894; Osborne and Campbell. Ibid. 19: 509. 1897; Osborne and Harris. Ibid. 25: 837. 1903.

⁴ Osborne and Harris. Amer. Jour. Physiol. 13: 436. 1905; Idem. Jour. Amer. Chem. Soc. 25: 323 and 837. 1903; Osborne and Campbell. Ibid. 19: 454. 1907; Osborne. Ibid. 24: 140. 1902; Osborne and Gilbert. Amer. Jour. Physiol. 15: 333. 1906; Osborne, Leavenworth and Brautlecht. Ibid. 23: 180. 1908.

⁵ Osborne. Amer. Chem. Jour. 14: 662. 1892; Idem. Zeitschr. f. physiol. Chem. 33: 240. 1901; Osborne and Harris. Jour. Amer. Chem. Soc. 25: 323, 474 and 837. 1903; Abderhalden. Zeitschr. f. physiol. Chem. 37: 499. 1902.

⁶ Osborne. Jour. Amer. Chem. Soc. 17: 429 and 539. 1895; Osborne and Campbell. Ibid. 22: 379. 1900; Osborne and Voorhees. Ibid. 15: 392. 1893; 16: 524. 1894; Osborne and Harris. Amer. Jour. Physiol. 17: 223. 1906; Osborne and Clapp. Ibid. 17: 231. 1906.

⁷ Osborne and Voorhees. loc. cit.; Osborne and Harris. Amer. Jour. Physiol. 13: 35. 1905; 17: 223. 1906; Idem. Jour. Amer. Chem. Soc. 25: 323 and 842. 1903; Osborne and Clapp. loc. cit.; Osborne, Leavenworth and Brautlecht. loc. cit.

⁸ Osborne. loc. cit.; Osborne and Clapp. Amer. Jour. Physiol. 19: 117. 1907; Osborne and Harris. Jour. Amer. Chem. Soc. 25: 323. 1903.

⁹ Chittenden and Osborne. Amer. Chem. Jour. 13: 453. 1891; 14: 20. 1892; Osborne and Clapp. Amer. Jour. Physiol. 20: 477. 1908; Osborne and Jones. Ibid. 26: 212. 1910; Osborne and Harris. Jour. Amer. Chem. Soc. 25: 323. 1903; Osborne. Ibid. 24: 140. 1902; Willcock and Hopkins. Jour. of Physiol. 35: 88. 1906.

IV. **Glutelins** (p. 266). *Glutenin* (gluten casein, plant fibrin)¹ in wheat seeds is the part of gluten that does not dissolve in alcohol. Coagulates when boiled. N = 17.5%, including 2.1% diamino acid N, 12% monoamino acid N and 3.3% ammonia N.

The following table illustrates the quantitative ratios of amino acids in plant proteins. The data is taken chiefly from analyses by Osborne and his associates.² It can be seen from the table, first, that various proteins (*e.g.*, legumin and vicillin) which were once regarded as identical on the basis of their solubility and other properties, differ from each other in the content of amino acids, and secondly, that in all the proteins analyzed some amino acids occur in large quantities and others in small amounts.

Cleavage products	Legumin in %	Vicillin in %	Phaseolin in %	Edestin in %	Leucosin in %	Gliadin in %	Hordein in %	Zein in %	Glutenin in %
Glycocoll.....	0.39	0.00	0.55	3.80	0.94	0.00	0.00	0.00	0.89
Alanine.....	1.15	0.50	1.80	3.60	4.45	2.00	0.43	} 9.02	4.65
Valine.....	1.36	0.15	1.04	present	0.18	0.21	0.13		0.24
Leucine.....	8.80	9.38	9.65	20.90	11.34	5.61	5.67	18.30	5.95
Proline.....	4.04	4.06	2.77	1.70	3.18	7.06	13.73	9.04	4.23
Phenylalanine ..	2.87	3.82	3.25	2.40	3.83	2.35	5.03	6.22	1.97
Aspartic acid ...	3.21	5.30	5.24	4.50	3.35	0.58	1.32	1.71	0.91
Glutamic acid...	18.30	21.34	14.54	14.00	6.73	37.33	43.20	26.17	23.42
Serine.....	0.53	?	0.38	0.33	undeter.	0.13	0.10	1.02	0.74
Cystine.....	undeter.	undeter.	undeter.	0.25	undeter.	0.45	undeter.	undeter.	0.02
Oxyproline.....	undeter.	undeter.	undeter.	2.00	undeter.	undeter.	undeter.	0.00	undeter.
Tyrosine.....	2.42	2.38	2.84	2.13	3.34	1.20	1.67	3.19	4.25
Arginine.....	11.06	8.91	4.89	14.17	5.94	3.16	2.16	1.35	4.72
Histidine.....	2.94	2.17	2.62	2.19	2.83	0.61	1.28	0.82	1.76
Lysine.....	3.99	5.40	4.58	1.65	2.75	0.00	0.00	0.00	1.92
Ammonia.....	2.12	2.03	2.06	2.28	1.41	5.11	4.87	3.64	4.01
Tryptophane ...	present	present	present	present	present	present	present	0.00	present
Total amount of cleavage prod- ucts in % of protein analyzed	63.18	65.44	56.21	75.90	50.32	65.80	79.59	80.48	59.68

Only the reserve proteins of seeds have been studied in detail. The proteins of the protoplasm have been studied almost not at all. Fungi are especially rich in proteins but there the proteins seem to occur in large complexes.³ They cannot be extracted by salt solutions nor by alkalies but only by strong acids.⁴

23. ANALYTICAL METHODS

In order to extract the reserve proteins, the seeds are finely divided, treated with ether to remove the fat if necessary and then extracted with the suitable solvent. A 10% solution of sodium chloride is often used for plant globulins. The extract is then dialyzed by placing it in a collodion tube (Fig. 36) which is immersed in a current of water. As the salt is washed out of the dialyzer, the globulin remains and is precipitated as an amorphous coagulum.

For complete hydrolysis, at least 500 g. of protein are dissolved in three times its weight of fuming hydrochloric acid or in ten times its weight of 25% sulphuric acid. The mixture is then boiled for 6 hours with fuming HCl or 16 hours with 50% H₂SO₄ in a reflux condenser,

¹ Osborne and Voorhees. *loc. cit.*; Osborne and Harris. *loc. cit.*; Osborne and Clapp. *Amer. Jour. Physiol.* 17: 231. 1906.

² Only the analysis of edestin is taken from Abderhalden. *Zeitschr. f. physiol. Chem.* 37: 499. 1902, with the exception of the determination of diamino acids (Kossel und Patten. *Ibid.* 38: 39. 1903) and of glutamic acid (Osborne and Gilbert. *Amer. Jour. Physiol.* 15: 333. 1906).

³ Iwanow, N. N. *Ber. russ. Akad. d. Wiss.* Vol. 397: 1918 (Russian).

⁴ Winterstein, E. *Zeitschr. f. physiol. Chem.* 26: 438. 1899; Winterstein und J. Hoffmann. *Hofmeisters Beitr. z. chem. Physiol. und Path.* 2: 404. 1902.

whereby part of the amino acids are generally lost by the formation of gums if carbohydrate is present in the solution and this is usually the case. The hydrochloric acid is removed by evaporating under reduced pressure and shaking with cuprous oxide. The sulphuric acid is precipitated with barium hydroxide. To obtain the tyrosine and diamino acids it is best to hydrolyze with sulphuric acid while the use of hydrochloric acid according to E. Fischer's method¹ is recommended for getting the monoamino acids. The hydrochloride of glutamic acid precipitates when the hydrochloric acid solution is evaporated. After it is removed, the solution of the other amino acids is evaporated to the consistency of syrup, absolute alcohol is added and gaseous hydrochloric acid is passed through the mixture. This produces an ester union of the ethyl alcohol and the carboxyl groups of the hydrochlorides of the amino acids. The hydrochloride ethyl ester of glycocoll precipitates in characteristic crystals with melting point of 144° . The filtrate is then evaporated under reduced pressure and the hydrochloride split off from the hydrochloride ester with potassium carbonate or sodium alcoholate (after neutralizing the solution). The ethyl ester of the amino acid is then extracted with ether and subjected to fractional distillation under reduced pressure, after removing the solvent. Four fractions are usually obtained: 1. at 60° and 12 mm.; 2. at 100° and 12 mm.; 3. at 100° and 0.1–0.5 mm.; 4. at 175° (oil bath) and 0.1–0.5 mm. The first fraction is largely glycocoll and alanine esters, the second and third are chiefly esters of valine, leucine and isoleucine, while the proline ester occurs in all three fractions. The fourth fraction contains the esters of serine, phenylalanine, aspartic and glutamic acid (if the latter was not entirely removed as the hydrochloride). The esters are saponified by boiling with water and the amino acids are then obtained as pure products. Refer to analytical manuals² for the separation of the single amino acids in each fraction. The preliminary fractionation according to Dakin³ is an excellent supplement to Fischer's method. When the mixture of amino acids is extracted with butyl alcohol, the monoamino acids are separated from the dicarboxylic acids (aspartic and glutamic) and from the diamino acids. Among those extracted by butyl alcohol, only proline is soluble in ethyl alcohol. In this way it is separated from the others and later does not contaminate the fractions obtained according to Fischer's method.

A special hydrolysis with sulphuric acid is carried out to prepare the tyrosine. The nearly insoluble tyrosine crystallizes out when the acid is removed. After the tyrosine is taken out, the residue can be used to prepare arginine, histidine and lysine which can be done quantitatively by Kossel's method.⁴ The mixture is precipitated with phospho-tungstic acid (this precipitates only diamino acids and ammonia), the precipitate decomposed with barium hydroxide and the ammonia drawn off under reduced pressure. The clear filtrate is acidified with sulphuric acid and treated with a boiling solution of silver sulphate which is added until a drop of the mixture on a watch glass gives a brown precipitate with barium hydroxide. The diamino acids are then fractionated by special methods which are described in manuals of biochemical methods.

To obtain the tryptophane and cystine, the protein must be broken up with enzymes in place of acids. First the cystine and then the tryptophane is precipitated by mercuric sulphate in the presence of 5% sulphuric acid. The cystine and the tryptophane crystallize

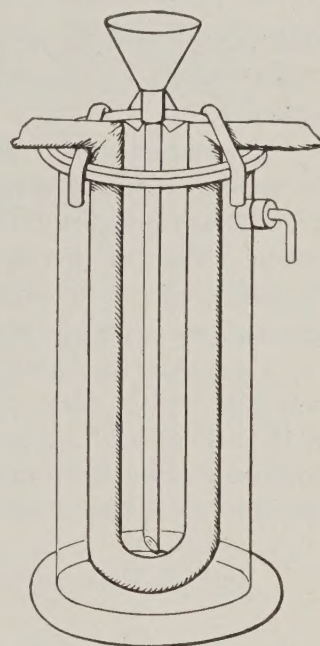


FIG. 36.—Dialyzer for proteins. Explanation in text. (After W. Kühne.)

¹ Fischer, E. *Zeitschr. f. physiol. Chem.* 33: 151. 1901.

² Abderhalden. *Handbuch d. biochem. Arbeitsmethoden.* 2: 470. 1910; *Idem.* *Neuere Ergebnisse auf dem Gebiete der speziellen Eiweisschemie.* 1909.

³ Dakin, H. D. *Biochem. Jour.* 12: 290. 1918.

⁴ Kossel, A. *Zeitschr. f. physiol. Chem.* 25: 177. 1898; 26: 586. 1898; Kossel und F. Kutscher. *Ibid.* 31: 165. 1900; Kossel und A. J. Patten. *Ibid.* 38: 39. 1903; Kossel und H. Pringle. *Ibid.* 49: 318. 1906; Weiss, E. *Ibid.* 52: 108. 1907; Steudel, H. *Ibid.* 37: 219. 1903; 44: 157. 1905.

The peptones are formed by digestion of protein with proteases; it is best to use gastric juice for 30 days at 40°. The proteins are then salted out and the peptones precipitated with ferric ammonium sulphate.¹ The proteins can be split into polypeptides without using enzymes. For this purpose the protein is exposed to a strong acid at 18°–37°. After removal of the acid, the complicated products are separated from the simpler ones with phosphotungstic acid: the latter can often be changed to esters and fractionated like amino acids. The large polypeptides are separated from each other in the form of β -sulpho-naphthalene derivatives or in the free condition. This often involves considerable difficulty.³ The nucleic acids are prepared from nucleoproteids,⁴ by extraction with dilute alkalis and sodium acetate at 100°. The nucleic acids are hydrolyzed by boiling with strong acids—sulphuric is the best. After hydrolysis, the liquid is freed of sulphuric and phosphoric acid by the use of barium hydroxide, and is precipitated with phosphotungstic acid. The precipitate contains purine bases and cytosine. After breaking up the precipitate, the bases are fractionated by special methods which are described in manuals of biochemical analysis.

24. PROTEIN TRANSFORMATIONS DURING THE GERMINATION OF SEED-PLANTS

The proteins in plants, just as in animals, are continually being broken down and regenerated. The plants differ from the animals in that there is no loss of nitrogen⁵ during all the transformations of proteins and the other nitrogenous substances in plants, while in animals there is a daily excretion of nitrogen from their bodies and this has to be supplied constantly. In the seed-plants, the proteins are synthesized in the leaves and carried by the vascular tissues to the other organs. Since the translocation of proteins requires their preliminary hydrolysis to crystalline products and since the proteins must be synthesized from amino acids, the latter must have a double origin. They can be either primary (from inorganic nitrogen and sugar) or secondary (from proteins). For this reason the question of protein transformations in the leaves is rather complicated and it is advisable to begin the description of transformations of nitrogenous substances in plants with the processes of seed-germination. In dormant seeds the nitrogenous food reserves are deposited almost entirely as proteins; hence crystalline nitrogenous products are formed in germination only by hydrolysis of the reserve proteins of the seed. This makes the scheme of transformation of nitrogenous substances simpler than in the leaves.

As mentioned above, most of the reserve proteins of seeds are globulins,⁶ while there are small amounts of albumins⁷ and some of the *Gramineae* have alcohol-soluble prolamines⁸ as well as glutelins.⁹ All these substances are deposited in the form of the so-called aleurone grains of various form and size. At the start of germination there is a considerable breaking down of protein.

¹ Siegfried, M. Zeitschr. f. physiol. Chem. 35: 164. 1902.

² Fischer, E. und E. Abderhalden. Ber. chem. Ges. 39: 752 and 2315. 1906; 40: 3544. 1907.

³ Abderhalden, E. Zeitschr. f. physiol. Chem. 58: 386. 1909.

⁴ Neumann, A. Arch. f. Anat. und Physiol. Suppl., p. 552. 1899; Herlant, L. Arch. f. exp. Path. und Phar. 44: 157. 1906; Slade, H. B. Amer. Jour. Physiol. 13: 464. 1905; Osborne und Harris. Zeitschr. f. physiol. Chem. 36: 85. 1902.

⁵ Boussingault. Agron., chim. agric. et physiol. 1: 1–249. 1864; 3: 266. 1864; 4: 245. 1868.

⁶ Ritthausen, H. Die Eiweisskörper der Getriedearten, Hülsenfrüchte und Ölsamen. 1872; Weyl, Th. Zeitschr. f. physiol. Chem. 1: 72. 1877; Palladin, W. Zeitschr. f. Biol. 31: 191. 1895.

⁷ Osborne and others, cf. p. 285.

⁸ Ritthausen, H. loc. cit.; Taddei. Schweig. Jour. 29: 514.

⁹ Osborne. P. 286.

If germination takes place in darkness there is more protein cleavage; in the light the green parts of the seedling soon begin to synthesize proteins.

The following figures show the extent of protein cleavage in darkness. If the total nitrogen in the seeds is taken to be 100, the quantity of protein nitrogen in the seeds and in the seedlings of various ages are expressed by the following figures (in mg.), taken from Prianischnikow:¹

	Seeds	Seedlings after			
		10 Days	20 Days	30 Days	40 Days
<i>Vicia sativa</i>	89.9	49.2	35.6	29.8	28.8

It can be seen from this that two-thirds of the total protein in the seed is broken down during germination in darkness. In some cases even four-fifths of the protein is split. When illuminated the protein cleavage continues for 10 to 20 days, depending on the species of plant. After this period there is an increase in the total amount of protein, as shown by the following data of Prianischnikow:²

	Seeds	Protein N in Mg. Seedlings after			
		7 Days	14 Days	21 Days	28 Days
<i>Phaseolus multiflorus</i>	82.7	52.6	68.2	73.6	70.8
<i>Lupinus luteus</i>	72.8	37.7	52.0	68.7	70.0

The proteins of the seeds are hydrolyzed by enzymes³ which are formed during germination⁴ and the proteins are broken down into crystalline products, *i.e.*, this is a "trypsin digestion." These enzymes work better in a slightly acid medium.⁵ It is not improbable that the proteolytic enzymes of seeds always consist of two compounds; protease, which splits proteins to peptones, and peptase which breaks the peptones and polypeptides into crystalline products (p. 280). Detailed study of proteolysis during the first stage of germination has shown that protein cleavage starts slowly, becomes faster and is finally retarded again. The curve of such a proteolysis is similar to the so-called grand curve of respiration and the grand curve of growth.⁶ The powerful processes of forming new tissues and organs are thus accompanied by a vigorous combustion of materials which supply energy, *i.e.*, the sugars, and by a marked protein cleavage. However, the splitting of protein is not a part of the respiration process as might be supposed at first glance. The coincident forms of the curves of the two processes only depends on the coördination

¹ Prianischnikow, D. Protein Cleavage During Seed Germination, p. 22. 1895 (Russian). Analogous results have also been obtained by many other investigators.

² Prianischnikow, D. The Proteins and Their Transformations in Plants, p. 75-77. 1899 (Russian). Seeds already germinated were used for this experiment.

³ Gorup-Besanez. Ber. chem. Ges. 7: 146 and 1478. 1874; 8: 1510. 1875; Green, J. R. Phil. Trans. Roy. Soc. London. 177: 39. 1887; Idem. Proc. Roy. Soc. London. 41: 466. 1886; 47: 146. 1890; 48: 370. 1891; Butkewitsch, W. The regressive metamorphosis of proteins in the seed-plants and the rôle of the proteolytic enzymes. 1904 (Russian); and others.

⁴ Abderhalden und Dammhahn. Zeitschr. f. physiol. Chem. 57: 332. 1908; Pugliese, A. Arch. di fisiol. 10: 292. 1912.

⁵ Butkewitsch. *loc. cit.*; Schjerning. Med. fra Carlsberg lab. 9: 319. 1913.

⁶ Prianischnikow, D. The proteins and their transformations in plants in relation to respiration and assimilation. 1899 (Russian).

between respiration and protein cleavage, because they are both part of the same great physiological process of germination. However, some have tried to develop a "protein theory" of respiration¹ on the basis of the hydrolysis of proteins during seed germination. According to this theory, only the "labile" living proteins of the protoplasm serve as respiratory material while the carbohydrates are used only to regenerate the protein. It must be emphasized that such views either lack a factual basis or were developed when the nature of the transformations of nitrogenous substances in plants had not been clarified. Now there is no doubt that the *reserve proteins of the aleurone grains and not the nucleoproteids of the protoplasm* are decomposed during germination. The amount of protein undigested by gastric juice (including the nuclein complexes) in general does not diminish during seed germination: it actually increases² because new living cells with their protoplasm and nuclei are formed in the young plants from the reserve protein. Still more convincing in this connection are the results obtained by determinations of protein phosphorus and of purine bases.³

The following table gives some analytical results obtained by determinations of undigested nitrogen (from Palladin) and nuclein phosphorus and purine nitrogen in seedlings (after Zaleski):

WHEAT

	Seeds	Seedlings after			
		3 days	6 days	9 days	14 days
Digested N.....	61.9	...	48.8		44.9
Undigested N.....	5.0	5.2	9.0	12.1	

Vicia faba, AXILLARY ORGANS

After	Purine N	Nuclein N	Protein N
3 days.....	0.0075	0.0125	
9 days.....	0.0262	0.0337	
3 days.....		0.0120	0.0849
9 days.....		0.0336	0.0376

From these results there can be no doubt that there is an increase in the protein of protoplasm and nuclei in young, growing parts of plants. In the

¹ Detmer, W. Jahrb. wiss. Bot. 12: 237. 1879; Idem. Vergleichende Physiologie des Keimungsprozesses der Samen. 1880; Idem. Ber. bot. Ges. 10: 443 and 442. 1892.

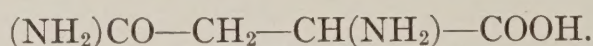
² Palladin. Rev. gén. bot. 8: 228. 1896; Idem. Arb. d. Warschauer Naturforscherges. 1898 (Russian).

³ Zaleski, W. Ber. bot. Ges. 25: 213. 1907; 29: 146. 1911; Idem. The transformations and the rôle of the phosphorus compounds in plants, p. 130-156. 1912 (Russian).

seeds, however, according to Zaleski's data, there are reserve nucleins which are split by the corresponding enzyme, the nucleases. The crystalline products of this cleavage pass to the young parts of the seedling where they are used in the synthesis of the proteins of protoplasm and the nuclei. The following interesting fact also shows that the protoplasmic proteins are not decomposed during seed germination but are actually synthesized rapidly. In leek bulbs both nitrogenous and nitrogen-free reserves are stored in the form of crystalline products and the reserve nitrogen is in the form of amino acids. As soon as the bulb begins to grow there is a rapid synthesis of protein.¹ There are almost no reserve proteins in this plant and hence there can be no appreciable proteolysis.

25. ASPARAGINE AND ITS PHYSIOLOGICAL RÔLE

On the basis of all the results of protein chemistry it might be expected that the cleavage of reserve proteins by enzymes during seed-germination must give the same products and in the same proportion as appear in the hydrolysis of protein by acids. The actual state of the case is complicated, for in seed germination there are formed large amounts of asparagine,² the half amide of aspartic acid:



There is a particularly intensive formation of asparagine during the germination of the legumes, the seeds of which contain large quantities of reserve protein. Oftentimes more than half of the protein in the seed of legumes changes to asparagine during germination. This is explained by Pfeffer's "asparagine theory"³ which was extended to other plants by Borodin.⁴ Borodin showed that asparagine would accumulate if the seedlings were placed in darkness. The theory said that since the plants are very economical with their nitrogen and never excrete it into the surrounding medium, asparagine plays the rôle of a reserve substance into which protein nitrogen is changed in order to move it about in the plant. During the assimilation of carbon in the light and the photochemical synthesis of carbohydrate, the asparagine is used to form protein in the growing parts of the plant but when carbohydrates are lacking the asparagine is held in reserve.

The basis of this theory is correct; asparagine is actually used to regenerate proteins in the light in the presence of carbohydrates but in some respects the theory is obsolete and needs to be modified. Among other things, Pfeffer claimed that protein passes directly into asparagine, *i.e.*, that protein is not split in the plant as in acid hydrolysis. This chemically improbable hypothesis was criticised by E. Schulze and his school. Schulze undertook detailed investigations of the formation and accumulation of asparagine and other products of physiological proteolysis, investigations which have been carried out with

¹ Zaleski, W. Ber. bot. Ges. 16: 146. 1898; 19: 331. 1901.

² Pasteur, L. Ann. de chim. et de physique (3) 31: 70. 1851; 34: 30. 1854; 38: 457. 1858; Boussingault. Compt. rend. 58: 881 and 917. 1864.

³ Pfeffer, W. Jahrb. wiss. Bot. 8: 530. 1872.

⁴ Borodin. J. Bot. Zeit. 1876.

unexcelled accuracy by the use of new methods which he worked out. These classical studies made the question of protein cleavage in seed germination one of the best-studied in the biochemistry of plants. The formation of asparagine during the splitting of protein by enzymes is nothing unusual because there are good reasons for thinking that the aspartic and glutamic acids which are found in protein hydrolysis by acids have lost their easily esterified amino group CONH_2 which as asparagine and glutamine they have in the protein molecule. An especially telling argument that reserve proteins contain the amides asparagine and glutamine rather than aspartic and glutamic acids is obtained by comparing the amounts of dibasic amino acids and ammonia produced in hydrolysis of protein by mineral acids.¹ If one calculates the quantity of ammonia split off from the amino groups of the dibasic amino acids and compares this value with that of ammonia found in protein hydrolysis, the result shows a remarkable likeness:

	Calculated	Found
Edestin.....	2.19%	2.28%
Globulin of cotton seeds.....	2.40%	2.33%
Conglutin.....	2.63%	2.55%
Zein.....	3.72%	3.61% etc.

Moreover it is found that in autolysis of seedlings the amount of amide nitrogen increases,² because the enzymatic hydrolysis of protein takes place without esterification of the amides.

Hence, it is most probable that there are amide residues in the protein molecule. However, the total amount of asparagine in seedlings cannot be a direct result of protein hydrolysis because the amount formed is much larger than the quantities of aspartic and glutamic acid in protein. The asparagine content often becomes 20% of the dry weight of the seedling.³ In a few cases there has been 25.7% asparagine found⁴ so that 73% of the nitrogen of the decomposed protein was recovered in the form of asparagine. Hence it is clear that asparagine must be synthesized at the expense of the other structural units of the protein molecule after it is hydrolyzed. This assumption was proved in E. Schulze's laboratory in the following manner. In some cases the formation of asparagine continues after protein cleavage has stopped whereupon the increase in asparagine corresponds exactly to the decrease in the other

¹ Osborne. *The Vegetable Proteins*. 1909; Osborne, Leavenworth and Brautlecht. *Amer. Jour. Physiol.* 23: 180. 1908.

² Butkewitsch, W. *The regressive metamorphosis of proteins etc.*, p. 55. 1904 (Russian); Schemtschuschnikow, E. *From the results of growth experiments and laboratory work of Prof. Prianischnikow* 11: 1. 1918 (Russian); Petrow, G. G. *The nitrogen assimilation of the seed-plants in light and in darkness*, p. 110. 1917 (Russian).

³ Schulze, E. and W. Umlauf. *Landwirt. Versuchs-Stationen*. 18: 1. 1875; and many others.

⁴ Schulze, E., Umlauf und Urich. *Landwirt. Jahrb.* 5: 821. 1876; Schulze, E. *Ibid.* 7: 411. 1878.

crystalline compounds of nitrogen.¹ This is shown by the following table which is taken from a paper by Schulze himself:

	For every 100 parts by wt. of ungerminated seeds			
	Protein N	Asparagine N	N from di-amino acids	N from other compounds
6 day seedlings.....	5.49	1.16	0.97	1.72
15 day seedlings.....	1.71	4.02	1.22	2.39
24 day seedlings.....	1.78	5.09	1.03	1.40

Protein cleavage ceased after 15 days but the asparagine continued to increase in amount, chiefly at the expense of the monoamino acids. Often-times no monoamino acids were found in etiolated seedlings of *Ricinus*,² evidently due to their use in forming asparagine, since there could be no synthesis of protein in the absence of light. Finally it was found that asparagine can be formed only in the presence of oxygen, although proteins in seedlings are split very rapidly in a medium free from oxygen.³ A supply of energy is required for the synthesis of asparagine and glutamine. This energy is obtained by the seed-plants chiefly in the processes of oxygen respiration. Prevention of oxygen respiration stops the synthesis of amides.

On the basis of various statements in the literature which have been considered very incompletely indeed on the preceding pages, it can be considered as established that only a small quantity of amides are formed directly by the hydrolysis of reserve proteins. Most of them are the result of synthesis at the expense of the other crystalline structural units of the protein molecule. Still a third method of forming asparagine has been discovered and this synthesis often supplies most of the amides in growing plants. It has been shown that asparagine can be formed from ammonia and carbohydrates⁴ by a direct primary synthesis, so that, for example, a considerable part of the inorganic nitrogen assimilated by plants from the substrate can change to asparagine at once. This emphasized the importance of calcium salts in the synthesis of asparagine and proved the unequal capacity of different plants to build asparagine directly

¹ Merlis, M. Landwirt. Versuchs-Stationen. 48: 419. 1897; Schulze, E. Zeitschr. f. physiol. Chem. 24: 18. 1898; Idem. Landwirt. Jahrb. 27: 503. 1898; cf. also Prianschnikow. The proteins and their transformations in the plant. 1899 (Russian).

² Schulze, E. Landwirt. Jahrb. 35: 621. 1906.

³ Palladin, W. The influence of oxygen on the protein cleavage in plants. 1889 (Russian); Idem. Ber. bot. Ges. 7: 126. 1889; Godlewski. Bull. de l'acad. des sciences de Cracovie. 1904.

⁴ Schulze, E. Landwirt. Jahrb. 17: 683. 1888; Idem. Zeitschr. f. physiol. Chem. 24: 18. 1898; Kinoshita. Bull. Univ. Tokyo. Vol. 2, No. 4. 1895; Suzuki. Ibid. Vol. 2, No. 7. 1897; cf. especially Prianschnikow and Schulow. Russ. Jour. f. exp. Landwirtschaft. Vol. 10. 1910 (Russian); Idem. Ber. bot. Ges. 28: 253. 1910; Idem. Rev. gén. bot. 25: 5. 1913.

from inorganic nitrogen and carbohydrates.¹ In the first category are plants which contain considerable quantities of carbohydrates (cereals) or fats; they synthesize amides from inorganic nitrogen in the absence of lime salts. In the second category are the legumes which form asparagine rapidly only in the presence of calcium salts. In the third category are plants which are extremely poor in carbohydrates (lupines): they synthesize practically no asparagine from carbohydrates and ammonia if the ammonium salts are physiologically acid.

In some plants, particularly the ferns and *Caryophyllaceae*, glutamine,² the half amide of glutamic acid $(\text{NH}_2)\text{CO}-\text{CH}_2-\text{CH}_2-\text{CH}(\text{NH}_2)-\text{COOH}$, is stored in place of asparagine. Indeed, there is no important difference between asparagine and glutamine. The primary synthesis of amides demands a revision of Pfeffer's idea of the physiological rôle of asparagine, as a substance which is designed to facilitate the translocation of protein nitrogen. On the basis of new results, Prianschnikow is abandoning Pfeffer's viewpoint and adopting in general the ideas of Boussingault³ who, with fine intuition, likened asparagine to urea in the animal organism. Both contain a store of ammonia which can easily be split off and used in synthetic processes. The animals continually excrete the urea and need new supplies of nitrogen every day while asparagine is stored in plants and its nitrogen is used as it is needed. It will be shown later that urea in some plants plays the rôle of asparagine. In this case the antithesis between the nitrogen exchange in animals and plants is still more marked because the fate of the same substance is entirely different in animals and in plants. Plants acquire their nitrogen in the hard struggle for existence (Chap. II) and even the smallest amounts of this valuable element are carefully preserved and utilized in the plant cells. On the contrary, the animal lives at the expense of prepared plant proteins, does not utilize them economically and squanders this substance which costs him nothing in the way of synthetic work.

26. THE OTHER NITROGENOUS CLEAVAGE PRODUCTS OF PROTEINS DURING GERMINATION

As mentioned previously, asparagine contains at best 70–80% of all the nitrogen from decomposed protein, even in the legumes in which it occurs in very large quantities after protein cleavage. The balance comes out in amino acids which are directly concerned in the structure of the protein molecule. In other plants there is more nitrogen of broken down proteins held in the pri-

¹ Prianschnikow. The proteins and their transformation. 1899 (Russian); Anniv. vol. for K. A. Timiriazeff, p. 241. 1916 (Russian); Smirnow. From the results of growth experiments and laboratory work of Prof. Prianschnikow. 9: 470. 1914 (Russian); Periturin. *Ibid.* 7: 180. 1912 (Russian); Prianschnikow. *Ibid.* 9: 559. 1919 (Russian); Nikolaewa. *Ibid.* 10: 380. 1916 (Russian); Ritman. *Ibid.* 7: 212. 1912 (Russian); Prianschnikow and Kaschewarowa. *Ibid.* 10: 174. 1916 (Russian).

² Schulze, E. Landwirt. Jahrb. 7: 431. 1878; Idem. Landwirt. Versuchs-Stationen. 47: 33. 1896; 49: 442. 1898; Idem. Zeitschr. f. physiol. Chem. 20: 327. 1894; Idem. Ber. bot. Ges. 25: 213. 1907; Steiger. Zeitschr. f. physiol. Chem. 86: 245. 1913.

³ Boussingault. Compt. rend. 58: 817. 1864; Idem. Agronomie, chimie agric. et physiol. 4: 264. 1868.

mary products, *i.e.*, in the monoamino and diamino acids. The proportions of individual amino acids in the seedlings do not correspond to their ratios in the reserve protein because the various amino acids are not used at equal rates in the formation of amides, regeneration of proteins and other processes. For example, alanine has never been isolated from germinating plants. Leucine¹ and tyrosine² are particularly common in seedlings. In addition, isoleucine³ (in legumes), valine,⁴ phenylalanine,⁵ proline and tryptophane,⁶ and the diamino acids—arginine,⁷ lysine and histidine⁸—have been demonstrated. Arginine was first discovered by Schulze in seedlings of the yellow lupine; this extremely important substance had not been previously known in chemistry. Later it was found that there is very much arginine in seedlings of the Conifers where its content is greater than that of all the other amino acids.⁹ This is shown in the following table, in which the numbers express the quantities of nitrogen in diamino acids and in the other crystalline products (including the amides) as percentages of the total nitrogen:

	N of diamino acids in %	N of the other cryst. products in %
Green seedlings of fir.....	21.5	4.7
Etiolated seedlings of pine.....	29.3	16.1
Pine under natural conditions.....	14.9	20.7

The amino acids are used partly to regenerate proteins in the growing parts of the plant and are partly decomposed by processes to be described later.

27. THE PROTEIN EXCHANGE IN DIFFERENT PARTS OF MATURE SEED-PLANTS

The above-described process of protein transformations in germinating seeds exceeds quantitatively all the other protein transformations in the plant; moreover, it has been especially well studied because it is so important in agricultural chemistry. However, protein transformations in other parts of plants have been studied far less accurately. In the underground reserve food containers—tubers, bulbs and rhizomes—there is in general the same cleavage of reserve

¹ Gorup-Besanez. Ber. chem. Ges. 7: 146 and 569. 1874.

² Idem. Ber. chem. Ges. 10: 781. 1877.

³ Schulze, E. und Winterstein. Zeitschr. f. physiol. Chem. 45: 38. 1905.

⁴ Schulze, E. Landwirt. Jahrb. 12: 909. 1884; Schulze und Barbieri. Jour. f. prakt. Chem. 27: 337. 1883; and others.

⁵ Schulze, E. *loc. cit.*; Schulze and Barbieri. *loc. cit.*; Schulze. Zeitschr. f. physiol. Chem. 11: 201. 1887; and others.

⁶ Schulze und Winterstein. Zeitschr. f. physiol. Chem. 45: 38. 1905.

⁷ Schulze und Steiger. Ber. chem. Ges. 19: 1177. 1886; and many others.

⁸ Schulze. Zeitschr. f. physiol. Chem. 28: 465. 1889; 47: 507. 1906; and many others.

⁹ Schulze. Zeitschr. f. physiol. Chem. 22: 435. 1896; [see Mothes, K. Planta. 7: 583-649. 1929 for recent experiments and a complete bibliography].

proteins as in seed germination. As soon as the tuber or bulb begins to sprout, many active enzymes appear in them and there is a mobilization of reserve substances. Asparagine plays the same prominent rôle as in seedlings.¹ It is often replaced by glutamine² in the underground reserve food containers of perennial plants. Of the other amino compounds, leucine,³ tyrosine⁴ and arginine⁵ predominate in the underground organs of perennial plants just as in germinating seeds. The same thing is true of the bark of trees during the mobilization of reserves in the spring. In the germination of pine pollen, no trace of asparagine or glutamine⁶ has been found.

There is a regrettable gap in the insufficiently investigated nature of leaf proteins.^x Even the extraction of proteins from the leaves involves unexpected and poorly understood difficulties.⁷ Although a large part of the protein of leaves is to be regarded as reserve material,⁸ no aleurone grains have been found there. On the other hand, a considerable amount of nitrogenous substances can be extracted from young leaves with water;⁹ these are evidently not contained in the protoplasm. Undoubtedly amino acids are always present in the leaves.¹⁰ Asparagine has been detected in them recently for the first time.¹¹ Urea is also very common in the leaves of various plants;¹² this will be discussed in more detail later.

It can hardly be doubted that the reserve materials in leaves are transformed in the same way as in germinating seeds.¹³ There is a particularly rapid drainage of nitrogenous products from the leaves when the fruit is ripening. At this time the nitrogen of the reserve proteins in the leaves is changed in part to amino acid nitrogen but largely to asparagine nitrogen and in this form passes out of the leaves. Analyses of the leaf blades and petioles of *Lupinus albus* gave the following results:¹⁴

¹ Schulze. Landwirt. Jahrb. 12: 909. 1884; Schulze und Barbieri. Landwirt. Versuchs-Stationen. 21: 63. 1878; and others.

² Schulze. Ber. chem. Ges. 10: 85 and 109. 1874; Steiger, A. Zeitschr. f. physiol. Chem. 86: 245. 1913; and others.

³ Schulze und Barbieri. Landwirt. Versuchs-Stationen. 24: 167. 1880; Schulze. Landwirt. Jahrb. 12: 909. 1884; and others.

⁴ Schulze und Barbieri. loc. cit.; Schulze. loc. cit.; Schulze und Engster. Landwirt. Versuchs-Stationen. 27: 357. 1881; and others.

⁵ Schulze. Zeitschr. f. physiol. Chem. 21: 43. 1895; Idem. Landwirt. Versuchs-Stationen. 59: 331. 1904; 60: 331. 1904; and others.

⁶ Schulze und Planta. Zeitschr. f. physiol. Chem. 10: 326. 1886. The pollen is poor in proteins and even in nitrogen as a rule. On this point cf. Kiesel. Zeitschr. f. physiol. Chem. 120: 85. 1922.

^x The water soluble proteins of leaves and shoots of the apple tree have been carefully studied by Thomas (Plant Physiol. 2: 55, 67, 109 and 245. 1927) whose work indicates the presence of a single protein or type of protein in leaves. Before the leaves fall the protein is moved to the branches and stored there over winter.—Ed.

⁷ Winterstein, E. Ber. bot. Ges. 19: 326. 1901.

⁸ Meyer, A. Ber. bot. Ges. 33: 373. 1915; 35: 658. 1917; 36: 508. 1918; Idem. Flora. 111: 85. 1918.

⁹ André, G. Compt. rend. 155: 1528. 1912; 158: 1812. 1914.

¹⁰ Grindley, J. and Slater. Jour. Amer. Chem. Soc. 37: 1778 and 2762. 1915; 38: 1425. 1916.

¹¹ Wassilieff, N. Jour. f. exp. Landwirtschaft. 6: 385. 1905 (Russian); Chibnall, A. C. Biochem. Jour. 18: 395 and 405. 1924; Mothes, K. Planta. 1: 472. 1926; [7: 583-649. 1929.]

¹² Fosse, R. Compt. rend. 155: 851. 1912; 157: 941. 1913.

¹³ Mothes, K. loc. cit.

¹⁴ Wassilieff, N. loc. cit.

Leaf blades	July 7	Sept. 5
Protein nitrogen.....	79.92%	77.86%
Non-protein nitrogen.....	20.08%	22.14%
Asparagine nitrogen.....	5.81%	6.96%
Leaf petioles		
Protein nitrogen.....	65.52%	68.45%
Non-protein nitrogen.....	36.48%	31.55%
Asparagine nitrogen.....	22.45%	15.41%

Thus at the time when fruit is maturing, the leaf is just like a seed that is being emptied with the difference that the proteins are newly synthesized in the leaves but only stored proteins are used in the seeds. Before the leaves turn yellow most of their nitrogenous substances are withdrawn.¹

The results of the investigations of seed maturation² may be regarded as an interesting supplement to the doctrine of the general importance of asparagine. It has been found that the same process goes on here as in germination but in the opposite direction. The seed is the place of regeneration of protein and the crystalline substances, *i.e.*, the primary amino acids and asparagine, pass in from the leaves where they were synthesized. The general principle of protein exchange in the plant thus consists in its hydrolysis in the places where it is first formed abundantly and in the reserve food reservoirs, in order that it may be translocated to the growing and ripening parts, whereupon a considerable part of the primary amino acids are changed to secondary products, asparagine and glutamine. All these crystalline substances are sources of nitrogen for the regeneration of protein in the growing and ripening parts of plants.³

28. THE CHEMICAL NATURE OF THE CLEAVAGE AND REGENERATION OF PROTEINS IN SEED-PLANTS

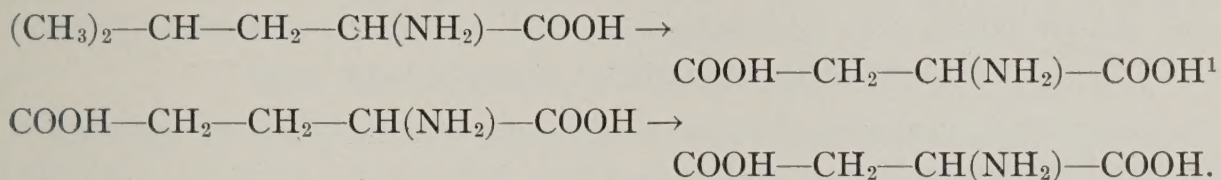
Since the ratio of amino acids in seedlings does not correspond to that in the protein molecule, as the result of the formation of asparagine and other transformations of the amino acids to be described later, different kinds of material transformations are necessary for the regeneration of proteins. For example, there can be no regeneration of protein with a shortage of carbohydrates because the frameworks of the structural units necessary to form proteins are synthesized from sugar. Theoretically there are two possible ways of forming asparagine after the reserve protein is hydrolyzed. In the first place, a few

¹ Combes, R. *Rev. gén. bot.* 38: 430, 510, 565, 632 and 673. 1926.

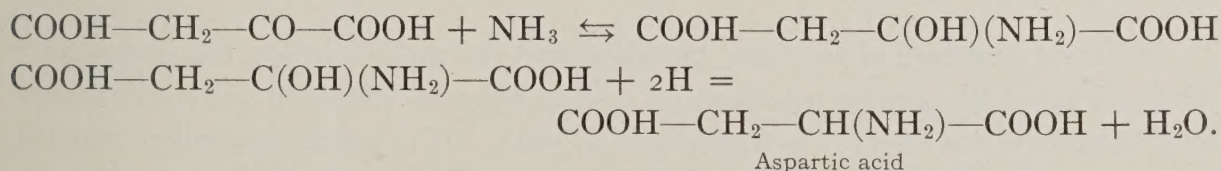
² Emmerling. *Landwirt. Versuchs-Stationen.* 24: 113. 1880; Nedokutschajew, N. *Mitt. d. Moskauer landwirt. Inst.*, p. 212. 1899 (Russian); Wassilieff, N. *Jour. f. exp. Landwirtschaft* 5 (I): 19. 1904 (Russian); Zaleski, W. *Ber. bot. Ges.* 23: 126 and 133. 1905; Idem. *Bieh. z. bot. Centralbl.* 27: 63. 1911; Schulze, E. *Landwirt. Jahrb.* 35: 621. 1906; Pfenninger, U. *Ber. bot. Ges.* 27: 227. 1909; Schulze und Winterstein. *Zeitschr. f. physiol. Chem.* 65: 431. 1909; Wassilieff, N. *Ber. bot. Ges.* 26: 465. 1908; Idem. *Protein synthesis in ripening seeds.* 1911 (Russian); Blagowestschensky. *Mitt. d. russ. Akad. d. Wiss.*, p. 423. 1916 (Russian).

³ Schulze gives a resumé of the most important results of the work of his school on this question in a summarizing article: *loc. cit.* [For a review of these and other problems of the protein metabolism of green plants, see Robinson, M. E. *New Phyt.* 28: 117-149. 1929. This summary includes specific items of evidence from the work of Kostychev, Prianischnikow, Chibnall, Kiesel, Schulze, Zaleski and others.]

amino acids, such as leucine and glutamic acid, can become aspartic acid by direct oxidation:



We already know (p. 286) that there are large amounts of glutamic acid and leucine among the structural units of the protein molecule, so that simple oxidation processes, which take no part in syntheses, might yield a considerable quantity of asparagine. Secondly, asparagine can be formed by transformation of amino acids by which ammonia is split off. In this case the asparagine framework is formed synthetically and only the nitrogen passes from the amino acids to the asparagine. Such a synthesis of asparagine is really identical with its primary synthesis from ammonia and sugar. The ammonia is incorporated in the molecule of malic acid $\text{COOH—CH}_2\text{—CHOH—COOH}$ or the corresponding ketonic acid, oxalacetic acid $\text{COOH—CH}_2\text{—CO—COOH}$, which is formed from sugar by oxidation. The latter hypothesis is more probable and the following facts support it. During the deaminization of amino acids, to be considered later, there are formed as intermediate products ketonic acids² which then either lose the carboxyl group adjacent to the ketone group or become reduced to hydroxy acids. This process takes place through the action of specific enzymes, the so-called deaminases, and it is very probably that the same enzymes act in the opposite direction under altered conditions and thus add ammonia to the molecule of ketonic acid:



It is possible that this is a general process of the primary formation not only of aspartic acid but of all amino acids. By experiments with isolated liver in which blood is passed through it, it is possible to obtain amino acids from ammonium salts of the α -ketonic or α -hydroxy acids.³ Such experiments with plants have been carried out only by indirect methods,⁴ yet it would be quite possible to use direct methods because the conditions for a synthesis of amino acids from sugar and inorganic nitrogen have already been found for the molds.⁵

The transformation of sugar into hydroxy or ketonic acids can be thought of in different ways. For example, Trier⁶ says that sugar can be simultaneously the material for the syn-

¹ By artificial oxidation, however, leucine loses the carboxyl group first and not the methyl groups.

² Neubauer, O. und Fromherz. *Zeitschr. f. physiol. Chem.* 70: 326. 1911.

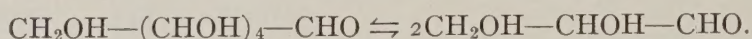
³ Embden, G. und E. Schmitz. *Biochem. Zeitschr.* 29: 423. 1910; 38: 393. 1912; Kondo, Kura. *Ibid.* 38: 407. 1912; Fellner. *Ibid.* 38: 414. 1912 (Papers from Embden's laboratory).

⁴ Prianischnikow, D. *Tageb. d. I Kongr. russ. Bot.*, p. 65. 1921 (Russian); Smirnow, A. *Ibid.*, p. 91; Idem. *Biochem. Zeitschr.* 137: 1. 1923.

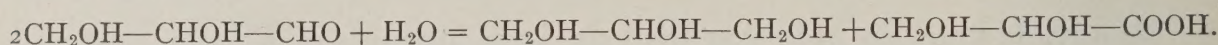
⁵ Kostytschew, S. und E. Tswetkova. *Zeitschr. f. physiol. Chem.* 111: 171. 1920.

⁶ Trier. Über einfache Pflanzenbasen und ihre Beziehungen zum Aufbau der Eiweissstoffe und Lecithine, p. 50. 1912.

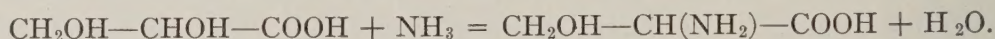
thesis of both fats and proteins (amino acids). Almost all theories of alcoholic fermentation assume that a three-carbon chain is formed in the first stage of the cleavage of sugar. The simplest process of this kind would be to split glucose into two molecules of glyceric aldehyde, a reaction the opposite of the aldol condensation which takes place in photosynthesis.



The glyceric aldehyde probably rearranges itself into glycerine and glyceric acid according to the Cannizzaro reaction:

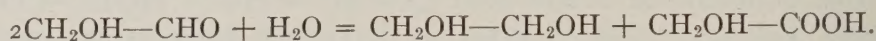


Glycerine is used to build fat but glyceric acid, by replacing the secondary hydroxyl group with ammonia, becomes the first amino acid—serine:

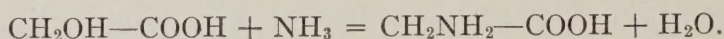


According to Trier, serine is said to yield alanine $\text{CH}_3-\text{CH}(\text{NH}_2)-\text{COOH}$ by reduction. It is more likely that alanine is formed from pyroracemic acid which can apparently arise from sugar in all plant cells.

According to Trier's view, glycocoll comes in the same way from glycolic aldehyde which is changed into glycol and glycolic acid:

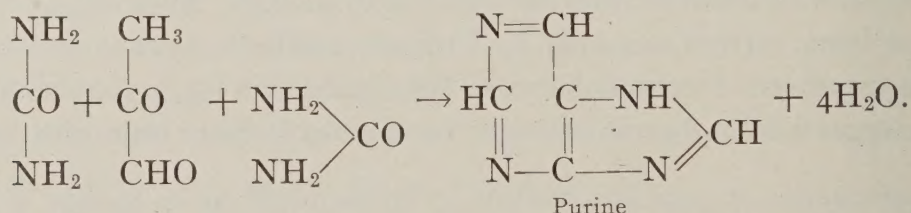


With ammonia, glycolic acid yields glycocoll:



In the same way glycol gives colamine $\text{CH}_2\text{OH}-\text{CH}_2\text{NH}_2$. The latter then becomes choline $\text{CH}_2\text{OH}-\text{CH}_2-\text{NOH}(\text{CH}_3)_3$ and is used in the formation of lecithin, the most important constituent of protoplasm. The origin of the five- and six-membered amino acids is less evident; it may be thought that they are formed by the lengthening of the carbon chain according to Strecker, including methylation. As for the cyclic amino acids, the closing of the aromatic ring in the living cell seems to be a special and still unsolved biochemical problem (Chap. VII), but the side chains of the cyclic amino acids are always alanine residues.

For the formation of arginine, histidine and the nuclein bases, use is probably made of urea, the primary formation and storage of which in plants has been established by the latest investigations (p. 306). It is usually assumed that the low content of urea in seed-plants is not due to lack of formation of it but to the rapid consumption of this product.¹ For example, the nucleus of purine might be synthesized from one molecule of methyl glyoxal $\text{CH}_3-\text{CO}-\text{CHO}$ and two molecules of urea:

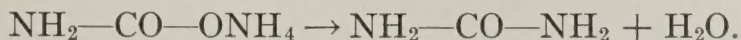


The synthesis of the pyrimidine nucleus requires one molecule of urea to one of methyl glyoxal. The latter is formed from sugar by the action of alkalies. Urea is readily formed by the oxidation of sugar, glycerine and formaldehyde in the presence of ammonia.² The intermediate product of this process is

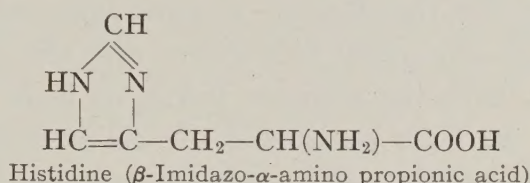
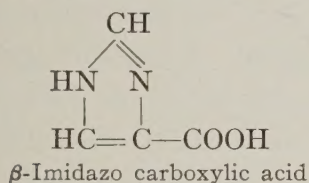
¹ Kiesel. *Zeitschr. f. physiol. Chem.* 75: 169. 1911; Trier. *loc. cit.*

² Fosse, R. *Compt. rend.* 154: 1187 and 1448. 1912; 155: 851. 1912; 156: 263 and 567. 1913; *Idem.* *Ann. de. chim. (IX)* 6: 13. 1916.

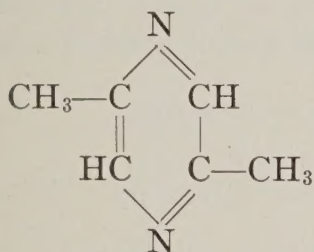
cyanic or isocyanic acid $\text{HO}-\text{C}\equiv\text{N}$ or $\text{HN}=\text{CO}$.¹ The enzyme urease, which hydrolyzes urea, also catalyzes the reverse process of forming urea from ammonium carbamate.²



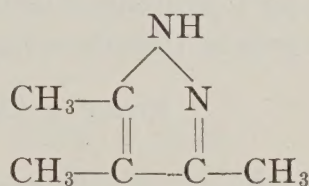
Of course, all these theoretical possibilities of the synthesis of nitrogenous substances in plants need experimental testing after which some of them might be omitted; just now they serve as working hypotheses. The extent to which the present organic chemistry is unable to explain the various reactions between ammonia and sugars can be seen from the following examples. In the action of an ammoniacal solution of cupric oxide on glucose, there is a formation of the imidazo ring and the result is β -imidazo carboxylic acids,³ the skeleton of which corresponds to that of histidine.



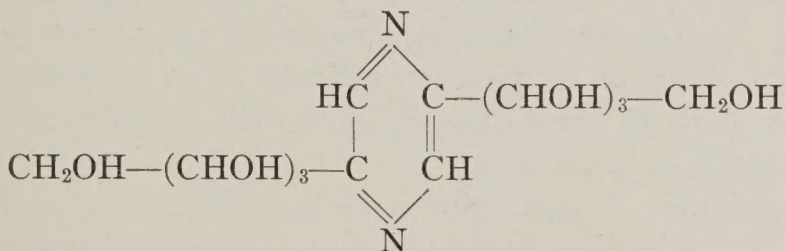
There is no support for the hypothesis that methyl glyoxal is formed as the intermediate stage in the synthesis of imidazo carboxylic acid. Under other conditions, the following cyclic substances⁴ have been obtained from ammonia and sugar:



or



or



The action of ammonium carbonate on sugar at a temperature of 30° gives compounds from which ammonia is partly released by boiling with dilute hydrochloric acid.⁵ These compounds are either amides, or ammonium aldehydes or perhaps osimines,⁶ *i.e.*, products of the attachment of ammonia to the hexose molecule; water is split off in this process (p. 238).

¹ Fosse, R. Compt. rend. 168: 320, 908 and 1164. 1919; 169: 91. 1919; *Idem.* Ann. Inst. Pasteur. 30: 1. 1916; 34: 715. 1920; Fosse et G. Laude. Compt. rend. 172: 684 and 1240. 1921; 173: 318. 1921; Fosse. *Ibid.* 173: 1370. 1921; Fosse et A. Hieulle. *Ibid.* 174: 39 and 1021. 1922.

² Mack, E. and D. S. Villars. Jour. Amer. Chem. Soc. 45: 501. 1923.

³ Windaus, A. und A. Ullrich. Zeitschr. f. physiol. Chem. 90: 366. 1914.

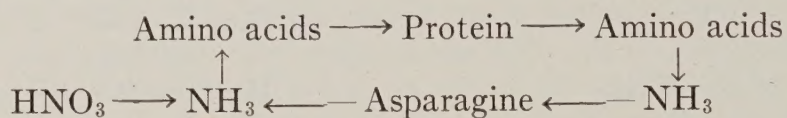
⁴ Dennstedt. Ber. chem. Ges. 25: 259. 1892; Bamberger und Einhorn. *Ibid.* 30: 224. 1897; Stoehr, C. *Ibid.* 24: 4105. 1891; *Idem.* Jour. f. prakt. Chem. 43: 156. 1891; 47: 439. 1893; Brandes, P. und C. Stoehr. *Ibid.* 54: 481. 1896; Lobry de Bruyn. Rec. des travaux chim. des Pays-Bas. 18: 72. 1899; Stolte. Biochem. Zeitschr. 12: 499. 1908; and others.

⁵ Kostytschew, S. und W. Brilliant. Jour. russ. bot. Ges. 5: 78. 1920 (Russian); *Idem.* Zeitschr. f. physiol. Chem. 127: 224. 1923.

⁶ Lobry de Bruyn. Rec. des travaux chim. des Pays-Bas. 12: 286. 1893; *Idem.* Ber. chem. Ges. 28: 3082. 1895; *Idem* und van Leent. Rec. des travaux chim. des Pays-Bas. 14: 134. 1895; 15: 81. 1896; Irvine. Biochem. Zeitschr. 22: 363. 1909; Fischer, E. und Leuchs. Ber. chem. Ges. 35: 3790. 1902.

It is quite conceivable that such substances become glucosamine or amino acids. Warming sugar with ammonium carbonate has already given products from which ammonia cannot be removed.

A further synthesis of protein from amino acids most probably takes place under the influence of the same enzymes which effect protein hydrolysis. The first step is probably the synthesis of polypeptides and the peptone-like substances,¹ corresponding to the double nature of the proteolytic enzymes which consist of peptone-forming proteases and the peptases (ereptases) which effect a hydrolysis of peptone to crystalline compounds. According to reliable data, in the regeneration of protein asparagine splits off ammonia which is used for syntheses² but the skeleton of asparagine takes no part in the formation of protein. This important fact shows that the regeneration of protein agrees in its chemistry with the primary synthesis from sugar and inorganic nitrogen compounds. In both cases there is an intermediate formation of ammonia and the skeleton of the amino acid is formed from sugar. It was pointed out above that the primary synthesis of protein and its regeneration are possible only in the presence of sugar. Thus the primary synthesis of protein starts from ammonia and protein decomposition stops when ammonia is formed. The nitrogen cycle in the plant has two intermediate stages of ammonia formation:



The part $\text{HNO}_3 \rightarrow \text{NH}_3 \rightarrow \text{Amino acids} \rightarrow \text{Protein}$ is the primary synthesis while $\text{Asparagine} \rightarrow \text{NH}_3 \rightarrow \text{Amino acids} \rightarrow \text{Protein}$ represents the regeneration of protein. On the other hand, asparagine also comes from protein through the intermediate stages of amino acids and ammonia. "Ammonia is the primary product and at the same time the end-product of the transformations of nitrogenous substances in the plant."³

The decomposition of asparagine with the splitting off of NH_3 was first produced in living plants by Butkewitsch (*loc. cit.*) by means of anaesthetics and pure cultures. Seedlings of yellow lupine poisoned with fumes of toluol were unable to bring about the synthetic process while the break-down of asparagine continued and was accompanied by an accumulation of ammonia.⁴ According to the same investigator, oxygen must be present in order that ammonia may accumulate.

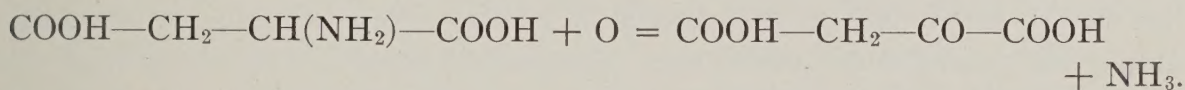
¹ Blagowestschensky. Jour. russ. bot. Ges. 4: 52. 1919 (Russian); *Idem.* Tageb. d. 1 Kongr. russ. bot., p. 21. 1921 (Russian); Biochem. Zeitschr. 168: 1. 1926.

² Butkewitsch. The decomposition of protein in the seed-plants and the rôle of the proteolytic enzymes, p. 98. 1904 (Russian); *Idem.* Biochem. Zeitschr. 16: 411. 1909; 41: 431. 1912.

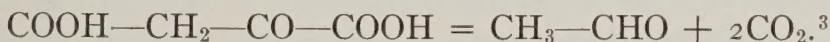
³ Prianschnikow, D. Anniv. vol. for K. A. Timiriazeff, p. 241. 1916 (Russian). [For a slightly different scheme from that given here by Kostychev, see Engel. Planta. 7: 133-164. 1929.]

⁴ According to Grover and Chibnall (Biochem. Jour. 21: 857-868. 1927) an enzyme in the rootlets of germinating barley will cause the deamidation of l-asparagine. Erepsins from pig intestine and from barley rootlets are both active toward asparagine. Hence, it is unnecessary to assume the presence of a specific asparaginase. Others, however, such as Bach, D. (Bull. Soc. Chim. Biol. 11: 995-1006. 1929) recognize asparaginase as a definite enzyme.—Ed.

The question involuntarily arises, what happens to the carbon framework of asparagine after the ammonia is removed? Aspartic acid has not been found¹ but this is not surprising because both the amide and the amine nitrogen is removed from the asparagine.² Considering that asparagine nitrogen often constitutes about 70% of the nitrogen of decomposed protein, it might be thought that the nitrogen-free asparagine residue must be present in large quantities and thus detected easily. Actually this residue cannot be found. In the deaminization of various amino acids by yeast, nitrogen-free substances of definite constitution are obtained but in this case also the aspartic acid disappears completely (see below). This puzzle needs to be studied experimentally. The writer of this book thinks that this question might be solved as follows. On the basis of the regularity with which amino acids are deaminized by yeast, it can be assumed that the first product of the removal of nitrogen from both asparagine and aspartic acid is oxalacetic acid.



This acid is then either reduced to malic acid or CO_2 is split off and it is changed to malonic acid. But before this process takes place most of the oxalacetic acid is split into acetaldehyde and carbon dioxide, according to the equation:



The acetaldehyde can be either oxidized further or reduced to alcohol; in either case it neither accumulates nor changes into stable compounds. Possibly the oxalic acid which is often formed during the cleavage and regeneration of proteins, is an oxidation product of the nitrogen-free residue of the asparagine.

Finally it is to be pointed out that the plants, unlike the animals, never form carbohydrates from proteins during protein cleavage under normal living conditions: at least there have been brought out no reliable facts which support such a transformation. Indeed it would not be expedient for plants to do this. Carbohydrates are the first products of organic synthesis by green plants and the resultant of the various chemical reactions in the plant cell is that various other compounds are synthesized from the carbohydrates (see next chapter), but the carbohydrates themselves are formed exclusively in the grand process of photosynthesis.

29. PROTEIN TRANSFORMATIONS IN THE LOWER PLANTS

The nature of the proteins and their transformations in the algae and mosses has not even been studied. However, a comparison of the nitrogen metabolism of the simplest green organisms, which do not need to transport protein, with that of seed-plants on the one hand and of plants without chlorophyll on the other, might be of special interest.

¹ Wassilieff. Jour. f. exp. Landwirtschaft. 6: 385. 1905 (Russian); Idem. Ber. bot. Ges. 26: 454. 1908; Schulze, E. Landwirt. Jahrb. 35: 621. 1906.

² Butkewitsch. loc. cit.; Petrow, G. G. Nitrogen assimilation by seed-plants. 1917 (Russian).

³ Neuberg, C. und L. Karczag. Biochem. Zeitschr. 36: 68. 1911.

According to available analytical data,¹ the protein content of bacteria is often unusually high (60–80% of the dry weight). In a few individual forms, such as the vinegar bacteria and *Azotobacter*,² the protein content is proportionally low (13–14% of the dry weight). The proteins of bacteria appear to be largely reserve materials. In general, when hydrolyzed they give the same products³ as those of proteins from seed-plants. However, it is noteworthy that no cystine appears in the bacteria. The structural units of the nucleins have also been isolated from bacteria.⁴ Nothing definite is known of the protein transformations in the bacteria. In view of the specialization of most bacteria, it is not improbable that this process is not the same in all species.

The proteins and their transformations in fungi have been investigated better although there are many gaps here in comparison with the seed-plants. In the cleavage of the proteins of the yeasts⁵ and molds⁶ with acids, the same amino acids have been obtained as in the cleavage of proteins from seed-plants but in the fungi, just as in bacteria, it has not been possible to find cystine among the products of hydrolysis. The nucleic acids of yeast have been very well studied (p. 277); in their chemical properties they are closely related to the other known nucleic acids. The proteins of the higher fungi differ sharply from those of the seed-plants in that they are not extracted by weak alkalies or salt solutions but are soluble in strong mineral acids.⁷ On the basis of recent information⁸ it can be assumed that most of the proteins of the higher fungi are glucoproteids; their carbohydrate group contains nitrogen and on hydrolysis yields so much glucosamine that it may consist entirely of this substance. "Plastin," which occurs in large quantities in the *Myxomycetes*⁹ and, like the proteins of the higher fungi, does not give a protein reaction unless previously split up, is probably such a glucoproteid, according to N. Iwanow. After removal of the carbohydrate group, the proteins of fungi may be split like the proteins of seed-plants and they furnish the same amino acids.¹⁰

Protein transformations have been studied best in the case of yeast. It has long been known that there is a vigorous cleavage of protein in yeast cells

¹ Nencki, M. Beitr. zur Biologie der Spaltspilze. 1880; Idem. Ber. chem. Ges. 17: 2605. 1884; Brieger. Zeitschr. f. physiol. Chem. 15: 134. 1891; Cramer. Arch. f. Hyg. 16: 151. 1892; 22: 167. 1895; and others.

² Alilaire, E. Compt. rend. 143: 176. 1906; Omeliansky, W. und N. Sieber. Zeitschr. f. physiol. Chem. 88: 445. 1913.

³ Tamura. Zeitschr. f. physiol. Chem. 87: 85. 1913; 89: 293. 1914; 90: 286. 1914.

⁴ Levene. Jour. Med. Research. 12: 251. 1904; Bendix, E. Deutsch. med. Wochenschr. 27: 18. 1901.

⁵ Ehrlich, F. Biochem. Zeitschr. 8: 410. 1908; Idem. Wochenschr. f. Braueri. 30: 561. 1913; Pringsheim, H. Ibid., p. 399; Thomas et Kolodziezka. Compt. rend. 156: 2024. 1913; 157: 243. 1913; 158: 1597. 1914; Idem. Bull. Soc. Chim. Biol. 1: 67. 1914; Meisenheimer, J. Wochenschr. f. Braueri. 32: 325. 1915; Idem. Zeitschr. f. physiol. Chem. 104: 229. 1919.

⁶ Thomas et Moron. Compt. rend. 159: 125. 1914; cf. also Malfitano. Ann. Inst. Pasteur. 14: 60 and 420. 1900; and others.

⁷ Winterstein, E. Zeitschr. f. physiol. Chem. 26: 438. 1899; Winterstein und Hofmann. Hofmeisters Beitr. chem. Physiol. und Path. 2: 404. 1902; Winterstein und Reuter. Centralbl. f. Bakt. (II) 24: 566. 1912; Idem. Landwirt. Versuchs-Stationen. 79–80: 541. 1913; Reuter, C. Zeitschr. f. physiol. Chem. 78: 167. 1912.

⁸ Iwanow, N. N. Mitt. russ. Akad. d. Wiss., p. 397. 1918.

⁹ Reinke, J. und Rodewald. Untersuch. d. bot. Lab. Göttingen. 1881.

¹⁰ Reuter, C. Zeitschr. f. physiol. Chem. 78: 167. 1912; Abderhalden und Rona. Ibid. 46: 179. 1905; Yoshimura, K. und Kanai. Ibid. 86: 178. 1913; and others.

during starvation and under the influence of antiseptics.¹ After the discovery of recent research methods, according to which it has been possible to press the cell sap out of yeast by the use of hydraulic presses, yeast was found to have proteolytic enzymes which split proteins directly to crystalline products.² A slightly acid reaction aids this action and an alkaline reaction retards it. Then followed a series of experimental data on the double nature of the proteolytic enzyme of yeast; that is to say, it consists of protease which forms peptones, and of peptase which splits peptones to crystalline products.³ The same amino acids are found in the proteolysis of yeast as appear in a typical trypsin digestion.⁴ The proteolytic enzymes of yeast are very powerful. In yeast juice the protein cleavage goes farther than it is known to for any other plant; 90% of the protein in yeast cells is decomposed in three days.⁵ It is very interesting that high concentrations of sugar and glycerine retard the action of the proteolytic enzymes of yeast.⁶

The significance of such a rapid cleavage of protein and of the presence of such powerful proteolytic enzymes in yeast is still unknown. In the seed-plants we find an intensive proteolysis only in cases of a mobilization of reserve proteins and their translocation to other organs. As to how far the reserve proteins of yeast cells on the one hand and of the proteins of the protoplasm on the other are broken down, is still unknown.

Not long ago it was thought that there is no protein cleavage in yeast cells with normal vital activity and rapid fermentation.⁷ However, the latest information shows that there is a protein cleavage under normal conditions.⁸ It must be borne in mind that investigations of microorganisms are always undertaken under conditions such that there are many millions of individuals taking part in the experiment. Under these conditions there are always a certain number of dead cells present. Possibly the proteins decompose only in the dead yeast cells while in the living yeast the proteolytic enzymes serve only for protein synthesis, since there is no doubt of the possibility of reversal of enzyme action (p. 40 ff.).

Sources of nitrogen for the synthesis of protein in yeast may include ammoniacal salts, amino acids and peptone preparations. The amino acids are partly used directly for the building of protein but only so far as its quantitative

¹ Thénard. *Ann. de chim. et de physique*. 46: 294. 1803; Pasteur, L. *Ibid.* 58: 407. 1860; Schützenberger. *Bull. Soc. Chim. de France*. 21: 204. 1874; Salkowski, E. *Zeitschr. f. physiol. Chem.* 13: 506. 1889.

² Hahn, M. *Ber. chem. Ges.* 31: 200. 1898; Geret und Hahn. *Ibid.* pp. 202 and 2335; *Idem.* *Zymasegärung*, p. 287. 1903; Salkowski. *Zeitschr. f. physiol. Chem.* 31: 305. 1900; 34: 159. 1901; Kutscher, F. *Ibid.* 32: 59 and 419. 1901; 34: 519. 1901.

³ Vines, S. H. *Ann. Bot.* 20: 113. 1906; 22: 103. 1908; 24: 213. 1910; Dernby, K. *Biochem. Zeitschr.* 81: 107. 1917; Iwanow, N. N. *Investigations of the transformations of nitrogenous substances in yeast*. 1919 (Russian); *Idem.* *Biochem. Jour.* 12: 106. 1918.

⁴ Kutscher. *loc. cit.*; and others.

⁵ Kostytschew, S. und W. Brilliant. *Zeitschr. f. physiol. Chem.* 91: 372. 1914.

⁶ Buchner, E., H. Buchner und M. Hahn. *Zymasegärung*, p. 316. 1903; Gromow, T. und O. Grigorjew. *Zeitschr. f. physiol. Chem.* 42: 299. 1904; Gromow, T. *Ibid.* 48: 87. 1906; Kostytschew und Brilliant. *loc. cit.*

⁷ Iwanow, L. A. *Zeitschr. f. physiol. Chem.* 42: 464. 1904.

⁸ Iwanow, N. N. *Investigations of the transformations of nitrogenous substances in yeast*. 1919 (Russian); *Idem.* *Biochem. Zeitschr.* 120: 25. 1921.

ratio in the solution corresponds to that in the yeast proteins.¹ Since it is inconceivable that the composition of the substrate would correspond exactly to that of the protoplasm, protein regeneration² in yeast, just as in the seed-plants, is accompanied by a reconstruction of amino acids. From the amino acids at hand, the nitrogen is split off as ammonia and used to build new amino acids—the ones necessary for protein synthesis. The same thing is found in the molds and a vigorous deaminization also takes place here. The difference from the seed-plants lies in that there is no intermediate formation of asparagine or other amides in the fungi. Since both yeast and molds tolerate ammonium salts very well, they have accumulations of ammonia, removed from the amino acids, in the form of salts of the various organic acids which are formed by the activities of the fungi.³ Especially large quantities of ammonia are found in the substrate of molds when the nutrient solution has an acid reaction but only when there is a shortage of sugar.⁴ In the presence of sugar the fungi grow rapidly and there is an intensive synthesis of protein which uses all the available ammonia. Hence the molds do not form a trace of ammonia in the presence of sugar in excess. Thus the transformations of nitrogen in the fungi are in general the same as those in the seed-plants. In both cases protein cleavage leads to the formation of amino acids and later to the removal of amine nitrogen in the form of ammonia; in both cases there is a synthesis of protein from ammonia and sugar. The only difference seems to be the absence in the fungi of the form of reserve nitrogen which is represented by asparagine and glutamine in the seed-plants. However, it will be shown later that there is an analogy in this respect between the green plants and those free from chlorophyll.

30. UREA AND ITS PHYSIOLOGICAL RÔLE

Urea was first found in the *Lycoperdaceae*⁵ and at first this was considered as an exception. Later it was discovered in various other fungi⁶ and, after the finding of a very sensitive test for urea, Fosse⁷ found it in seed-plants as well as in various fungi. After he had detected urea even in pure cultures of molds it became clear that urea is formed within the fungi themselves and is not taken in from the soil in that form, as earlier workers assumed. Among the seed-plants, those with mycorrhiza (p. 197 ff.) in their roots are notable for their content of urea.⁸

¹ Zaleski und Israilsky. Ber. bot. Ges. 32: 472. 1914.

² The "regeneration" here is a summarized one, so to speak, since it must be considered that every experiment involves a large number of organisms. Really we have no right, in connection with protein transformations in microorganisms, to speak of protein regeneration in the sense that it occurs in the seed-plants.

³ Butkewitsch, W. Jahrb. wiss. Bot. 38: 147. 1902; Idem. Anniv. vol. for K. A. Timiriazeff, p. 457. 1916 (Russian).

⁴ Butkewitsch, W. loc. cit.; Kostytschew, S. und Zwetkowa. Zeitschr. f. physiol. Chem. 111: 171. 1920.

⁵ Bamberger, M. und A. Landsiedl. Monatsch. f. Chem. 24: 218. 1903; 26: 1109. 1905.

⁶ Gaze, R. Arch. d. pharmacol. 243: 78. 1905; Goris, A. et M. Mascré. Compt. rend. 147: 1488. 1908; 153: 1082. 1911.

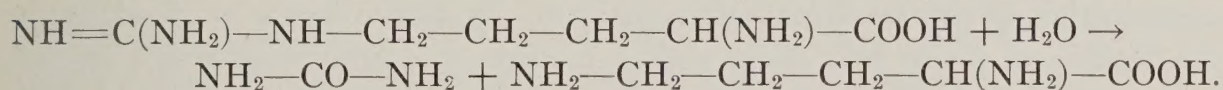
⁷ Fosse, R. Compt. rend. 154: 1187 and 1448. 1912; 155: 851. 1912; 156: 263 and 567. 1913; Idem. Ann. Inst. Pasteur. 30: 1. 1916; 34: 715. 1920; Idem. Ann. de chim. et de physique (IX) 6: 13. 1916.

⁸ Weinland, H. Jahrb. wiss. Bot. 51: 1. 1912; Tanböck, K. Öster. bot. Zeitschr. 76: 43. 1926.

In so far as it is possible, larger amounts of urea accumulate in fungi than in the seed-plants. The following table gives an idea of the maximum quantities of urea which have been found in some fungi.¹

Fungus	Urea in per cent of dry weight
<i>Lycoperdon saccatum</i>	2.85
<i>Lycoperdon piriforme</i>	4.62
<i>Lycoperdon gemmatum</i>	10.70
<i>Lycoperdon molle</i>	9.22
<i>Lycoperdon marginatum</i>	5.84
<i>Lycoperdon echinatum</i>	1.16
<i>Bovista nigrescens</i>	11.16
<i>Psalliota campestris</i>	6.18
<i>Psalliota pratensis</i>	1.61
<i>Pholiota spectabilis</i>	2.45
<i>Cortinarius violaceus</i>	0.51

Urea may have a two-fold origin. The amino acid arginine is broken down by the enzyme arginase and urea and ornithine are formed (see p. 270-271).



Arginase was first found in the liver and other animal organs² but it was soon detected in plants as well.³ In view of the wide distribution of arginase, the general occurrence of urea is satisfactorily explained.

Still the removal of urea from arginine does not account for the enormous accumulation of this product, which is evident from the preceding table. These amounts of urea are far greater than those of arginine in the protein of the fungi concerned. The primary formation of urea from ammonia was established by the latest experiments of Iwanow,⁴ in which the fruiting bodies of different species of *Lycoperdon* contained ammonia either as a gas or in the form of soluble salts of organic acids. Under these conditions there was an increase in the formation of urea.

Since in *Lycoperdon*, the urea gradually accumulates up to the time when the spores are maturing and is quickly consumed when they are ripe,⁵ it is entirely justifiable to regard it as a nitrogenous reserve material, similar to asparagine and glutamine of seed-plants; where urea is very common but does not accumulate in very large amounts, it is probably due to its rapid consumption.⁶ Urea is an excellent reserve material which splits off ammonia easily and can

¹ Iwanow, N. N. Biochem. Zeitschr. 136: 1. 1923; figures of the same order are given by Goris, A. et P. Costy. Compt. rend. 175: 539. 1922.

² Kossel, A. und Dakin. Zeitschr. f. physiol. Chem. 41: 321. 1904; 42: 181. 1904.

³ Shiga. Zeitschr. f. physiol. Chem. 42: 505. 1904; Kiesel, A. Ibid. 75: 169. 1911; 118: 267. 1922; Idem. Arginine and its transformations in plants. 1916 (Russian).

⁴ Iwanow, N. N. Biochem. Zeitschr. 150: 115. 1924; 181: 1. 1927.

⁵ Iwanow, N. N. Biochem. Zeitschr. 135: 1. 1923; loc. cit.

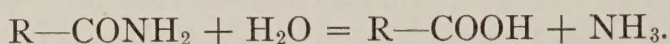
⁶ Kiesel, A. Zeitschr. f. physiol. Chem. 75: 169. 1911.

also be used directly for the synthesis of the most important substances, such as arginine, histidine and the purine and pyrimidine bases (see above).

Urea is split in plants by means of the enzyme urease which was first found in the bacteria which ferment it.¹ Urease is very widespread in plants and often accompanies urea.² In this respect there is a difference between plants and animals which contain no important quantities of urease and hence excrete urea with all its nitrogen. On the contrary, the plants without chlorophyll employ urea as a reserve material and can use it for protein synthesis. The behavior of urea is entirely analogous to that of asparagine in seed-plants. Urea is formed only in the presence of oxygen and it accumulates in large amounts especially when there is a shortage of carbohydrate.³ At this opportunity the fundamental difference between the nutrition of animals and the plants without chlorophyll must be emphasized again. The erroneous but widespread view was once held that the plants with no chlorophyll are nourished like animals.

31. DEAMINIZATION AND OTHER ALTERATIONS OF AMINO ACIDS IN PLANTS

On the preceding pages there have been several references to deaminization of amino acids, *i.e.*, the removal of the amino group. It takes place in the regeneration of protein from asparagine and glutamine in the seed-plants, in the feeding of molds and yeast with a mixture of amino acids which do not correspond exactly to the proportion of the amino acids in the proteins of these plants, and very likely in the various processes associated with the formation and accumulation of urea. The amide group of amides is removed hydrolytically by specific enzymes, the so-called deamidases. According to present-day ideas, the amino group is removed by different enzymes, the deaminases, with the assistance of oxidation processes.⁴ The deamidases change the amide group to the free carboxyl group:



Deaminization, *i.e.*, the removal of NH_3 from the amino group, has been studied best for yeast. It has been known for a long time that in alcoholic fermentation there is a formation of fusel oils, *i.e.*, monovalent alcohols of various structure, including some that are optically active. Only recently has it been proved that the fusel oils come directly from the amino acids.⁵ The structure of the fusel oils corresponds completely with that of the amino acids from which they have arisen. According to Ehrlich's investigations, there is a definite regularity in the process of changing the amino acids into

¹ Miquel, P. Compt. rend. 111: 397. 1890. [The distribution of urease in plants is discussed by Wasicky and Krach. Österr. Bot. Zeitschr. 77: 271-279. 1928.]

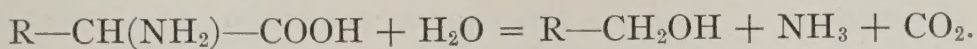
² Kiesel, A. Arginine etc. 1916; Kiesel und Troizky. Zeitschr. f. physiol. Chem. 118: 247. 1922.

³ Iwanow, N. N. Biochem. Zeitschr. 154: 376 and 391. 1924; 162: 425. 1925; 201: 1. 1928.

⁴ Butkewitsch, W. Anniv. vol. for K. A. Timiriazeff, p. 457. 1916 (Russian).

⁵ Ehrlich, F. Ber. chem. Ges. 39: 4072. 1906; 40: 1027 and 2538. 1907; 44: 130. 1911; 45: 883. 1912; Idem. Zeitschr. d. Ver. d. Zuckerindus. 55: 539. 1905; Idem. Biochem. Zeitschr. 2: 52. 1906; Idem. Landwirt. Jahrb. 38 (Erg.-bd. V): 289. 1909; Idem. Biochem. Zeitschr. 63: 579. 1914; 73: 232. 1917.

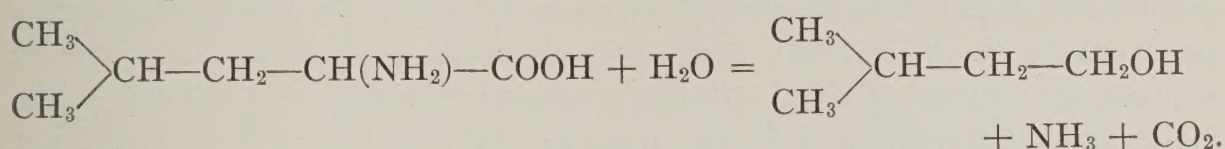
products free from nitrogen. The monoamino acids give rise to the monovalent alcohols in the following manner:



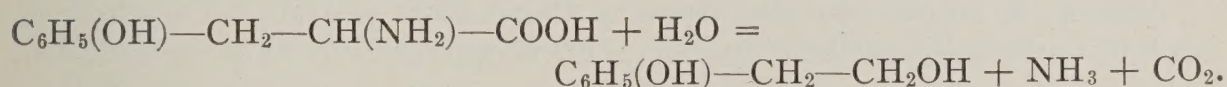
Actually this reaction consists of four stages:¹

- I. $R-CH(NH_2)-COOH + O = R-COH(NH_2)-COOH$ (oxidation)
- II. $R-COH(NH_2)-COOH = R-CO-COOH + NH_3$ (deaminization)
- III. $R-CO-COOH = R-CHO + CO_2$ (the removal of CO_2)
- IV. $R-CHO + 2H = R-CH_2OH$ (reduction).

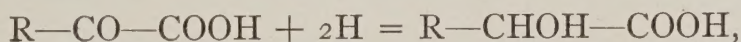
The apparent addition of water is thus actually a combination of oxidation and reduction. This condition is quite common in biochemistry. Isoamyl alcohol is formed from leucine by the foregoing reactions:



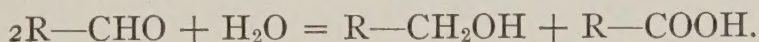
The aromatic alcohol tyrosol is formed from tyrosine:



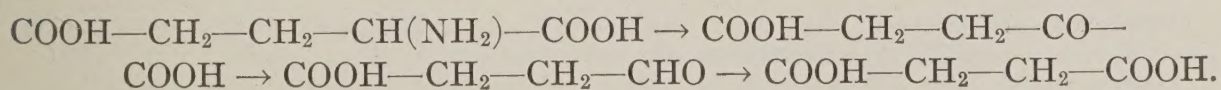
The corresponding alcohols are obtained in the regular way from the other amino acids. A considerable quantity of by-products results because there is either a direct reduction of ketonic acids, without removal of the carboxyl group



or part of the aldehyde is not reduced to alcohol but is oxidized to an acid (or transformed according to the Cannizzaro reaction).



Histidine undergoes exactly the same change as tyrosine. Arginine and lysine have not yet been investigated in this respect. It is interesting that there is an asymmetrical cleavage of amino acid: if yeast is offered an optically inactive synthetic product, it splits only the optically active substance which is found in the protein molecule and leaves the other stereo-isomeric compound intact.² This is one method of preparing optically active amino acids for by the aid of the Walden inversion (p. 20), every optically active amino acid can be converted into the enantio-isomeric form. The dibasic amino acids are deaminized by yeast according to another scheme: the intermediate aldehyde is not reduced to alcohol in this case but is oxidized to an acid. For example, glutamic acid is thus changed to succinic acid:³



¹ Neubauer, O. und Fromherz. Zeitschr. f. physiol. Chem. 70: 326. 1911.

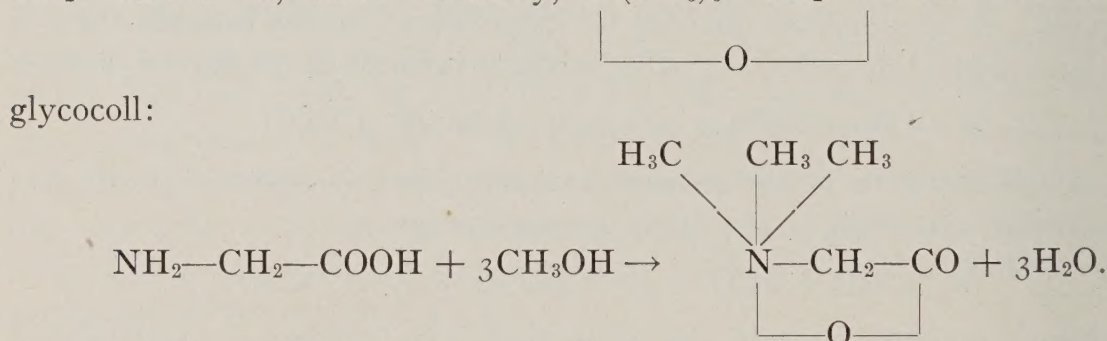
² Ehrlich, F. Biochem. Zeitschr. 1: 8. 1906; 8: 438. 1908; 63: 379. 1914.

³ Ehrlich, F. Biochem. Zeitschr. 18: 391. 1909 and papers cited above; Neubauer, O. und Fromherz. *loc. cit.* C. Neuberg has proved by experiments the intermediate formation of α -keto-glutaric acid $COOH-CH_2-CH_2-CO-COOH$: Biochem. Zeitschr. 91: 131. 1918.

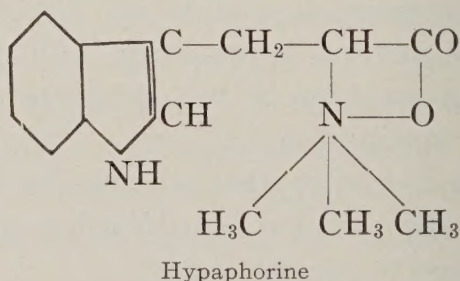
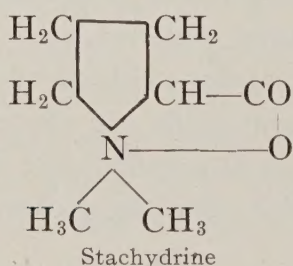
The product of deaminization of aspartic acid has not been isolated. The possible explanation of this phenomenon was given on p. 303. The whole process of formation of fusel oils and succinic acid from amino acids by yeast aims to obtain ammonia for the building of proteins. Ehrlich has shown that the "fermentation of amino acids" can be much suppressed by placing a considerable quantity of ammonium salts at the disposal of the yeast. On the other hand, a certain amino acid can be the source of nitrogen for yeast and then only a certain alcohol is formed. For the biochemist it is worth noticing that the example of the formation of fusel oils and succinic acid explains the capacity of plants to produce various kinds of *nitrogen-free* compounds from the structural units of the protein molecule.

The molds split amino acids in another way. No such regular course of deaminization has been observed in them as in yeast. In general, the amino acids change to the hydroxy acids of corresponding structure and therefore without the removal of CO_2 .¹ Unlike yeast, this reaction predominates in the molds and the formation of alcohol together with removal of NH_3 or CO_2 is a secondary process.

Among the noteworthy alterations of amino acids in seed-plants, which do not lead to the regeneration of protein, there is deaminization² and methylation, which results in the formation of a betaine.³ Ordinary betaine⁴ $\text{NOH}(\text{CH}_3)_3\text{—CH}_2\text{—COOH}$ or, more correctly, $\text{N}(\text{CH}_3)_3\text{—CH}_2\text{—CO}$ is obtained from



Stachydrine, the betaine corresponding to this amino acid, is obtained from proline (pyrrolidine-carboxylic acid) in the same way. From tryptophane also comes its corresponding betaine, namely hypaphorine.⁵



¹ Ehrlich, F. und Jacobsen. Ber. chem. Ges. 44: 888. 1911; Ehrlich, F. Biochem. Zeitschr. 36: 447. 1911 and papers cited above; Pringsheim, H. Biochem. Zeitschr. 3: 121. 1907; 8: 128. 1908.

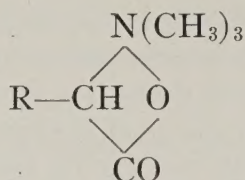
² Oparin, A. Biochem. Zeitschr. 124: 90. 1921.

³ Schulze, E. und G. Trier. Ber. chem. Ges. 42: 4654. 1909; Idem. Zeitschr. f. physiol. Chem. 59: 233. 1909; 67: 46 and 59. 1910; 76: 258. 1911; 79: 235. 1912; Yoshimura, K. und Trier. Ibid. 77: 290. 1912; Deleano, N. und Trier. Ibid. 79: 243. 1912.

⁴ Stolzenberg, H. Zeitschr. f. physiol. Chem. 92: 445. 1914.

⁵ Romboutgh and Barger. Trans. Chem. Soc. 99: 2068. 1911.

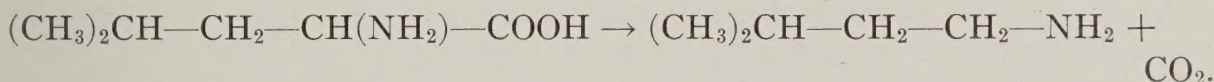
In general the betaines correspond to the amino acids $R-CH \begin{cases} NH_2 \\ COOH \end{cases}$



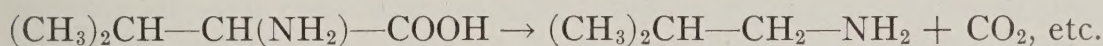
However, there is not always this regularity and the amino acids often undergo other changes beside methylation. Then there are formed various nitrogenous bases which will be considered in the next chapter.

In addition to these transformations, the amino acids in plants are often decarboxylized and changed to the corresponding amines.¹ This process is observed frequently in the bacterial decomposition of amino acids.

Isoamyl amine is formed from leucine:

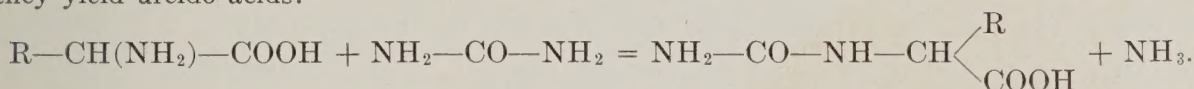


From valine comes isobutyl amine:

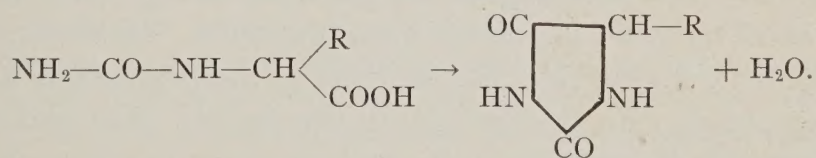


Most of the amino acids in seed-plants are actually deaminized for the synthesis of protein and only a very small part of them are changed to betaines, bases, etc. It has not yet been made clear whether the deaminization in seed-plants is analogous to that of yeast or molds.

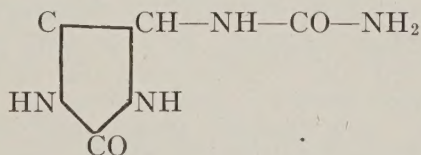
In addition to the above-described transformations, the amino acids themselves, without the assistance of enzymes, form compounds with a few substances. For example, with urea they yield ureido acids:²



The ureido acids readily split off water and close up to cyclic hydantoins:



For example, one of these hydantoins is allantoin,³ which is repeatedly found in plants:



Allantoin

¹ Barger, G. and Dale. Proc. Chem. Soc. 26: 128. 1910; Idem. Jour. Chem. Soc. (London) 97: 2592. 1910; Reuter. Zeitschr. f. physiol. Chem. 78: 167. 1912; and others.

² Lippich. Ber. chem. Ges. 39: 2953. 1906; 41: 2958 and 2976. 1908; Weiland. Biochem. Zeitschr. 38: 385. 1912; Kondo. Ibid. 38: 411. 1912.

³ Schulze, E. und Barbieri. Ber. chem. Ges. 14: 1602. 1881; Idem. Jour. f. prakt. Chem. 25: 145. 1882; Idem. Zeitschr. f. physiol. Chem. 11: 420. 1886; Idem. Landwirt. Jahrb. 21: 105 1892; Schulze, E. und Bosshard. Zeitschr. f. physiol. Chem. 9: 420. 1886.

However, allantoin is probably formed from urea and a nitrogen-free substance, without any connection with the amino acids. It is prepared synthetically from two molecules of urea and one of glyoxylic acid $\text{COOH}-\text{CHO}$.

Another process which probably takes place in living plant cells is the union of amino acids with simple sugars. This occurs even at rather low temperatures.¹ At first the sugar unites with the amino acid in such a way that either can be regenerated from the compound. Later there may be a condensation and the final product is a substance which is close to the humins and from which the nitrogen can no longer be removed.

The formation of dark colored substances, relatively poor in nitrogen and very resistant to acids and alkalis, takes place to a considerable degree in all media which are rich in both sugars and amino acids. This fact may be important in explaining the formation of humus in the soil, since plant residues are broken down there and it is inevitable that the amino acids come in contact with sugars. It is of interest that the products of the union of amino acids with glucose are made use of in Stutzer's method for the determination of protein nitrogen. That this can have a serious error is evident from the following. The linkage of amino acids with sugar also takes place at low concentrations of sugar.² Such reactions undoubtedly accompany various biological processes. For example, it has been known for a long time that only part of the nitrogen of the amino acids released in the protein cleavage in yeast, can be used for the regeneration of protein; the rest of it disappears. This brings up the subject of the "nitrogenous excrement" of fermentation and the alleged uneconomical use of nitrogen by the lower organisms.³ However, it was later found that the "excrements of fermentation" are products of the reaction between amino acids and sugar which takes place without the cooperation of enzymes and living protoplasm. According to Stutzer, the capacity of this substance to precipitate allows it to be confused with protein and leads to the question as to whether the protein of yeast is not decomposed during fermentation.⁴ Maillard⁵ claims that amino acids readily change to polypeptides (first to diketopiperazines) in the presence of sugar or glycerine. According to his conception, polypeptides can be synthesized in living cells by the intermediate stages of forming esters of sugar and glycerine.

32. THE PROTEINS OF THE PROTOPLASM

It is evident from the foregoing exposition that we must distinguish sharply between the reserve proteins and the proteins of the protoplasm. Confusion on this point has given rise to serious errors. The break-down of reserve proteins, which is involved in the general mobilization of reserve materials, has erroneously been taken as a change in the protoplasmic proteins which is said to accompany all of the most important vital functions. However, we have no concrete reasons for thinking that the proteins of the protoplasm are unstable and easily decomposed. In the seed-plants the protein cleavages which have so far been thoroughly investigated refer exclusively to reserve proteins. The absence of aleurone grains and other definite structures in the plants without chlorophyll makes it somewhat difficult to distinguish between the reserve proteins and the protoplasmic proteins in the cells of the fungi and bacteria.

¹ Kostytschew, S. und W. Brilliant. *Zeitschr. f. physiol. Chem.* 91: 372. 1914; 127: 224. 1923; *Idem.* *Mitt. russ. Akad. d. Wiss.*, p. 953. 1916 (Russian); *Idem.* *Jour. russ. bot. Ges.* 5: 71 and 78. 1920 (Russian); Grünhut und Weber. *Biochem. Zeitschr.* 121: 109. 1921.

² Iwanow, N. N. Investigations of the transformation of nitrogenous substances in yeast, 1919 (Russian).

³ Mayer, A. Untersuchungen über die alkoholischer. Gärung. 1869; Rubner. *Die Ernährungsphysiologie der Hefezelle bei alkoholischer Gärung.* 1913.

⁴ Iwanow, N. N. *loc. cit.*; *Idem.* *Biochem. Zeitschr.* 120: 1. 1921.

⁵ Maillard. *Compt. rend.* 153: 1078. 1911; 154: 66. 1912; 155: 1554. 1912; 156: 1159. 1913; *Idem.* *Genèse des matières protéiques et des matières humiques.* 1913. Maillard's syntheses differ from those of Kostychev and Brilliant; cf. Kostytschew and Brilliant. *loc. cit.*

We also have reasons for thinking that it is largely if not exclusively the reserve proteins which are split and deaminized. This is evident from the following consideration. The proteins of the protoplasm occur in an almost latent condition; they give no characteristic tests, are not isolated from the tissues by the usual methods of extracting proteins, and in a word they occur in a fixed condition, so to speak. In all cases in which we have to do with protoplasmic organizations, we meet with complicated nucleo- or glucoproteids. These may be only cleavage products of still larger complexes which include still other structural units of various constitution in addition to the carbohydrate groups and the purine and pyrimidine nuclei. This may be the key to the explanation of the regulation and correlations of the different biochemical processes. Wundt¹ thinks that these gigantic complex molecules—conglomerates of chemically different members—represent the simplest protoplasm. This question will be considered in more detail in the chapter on the systematic development of organs. If the protoplasmic proteins in the living cells involve different groups, it is scarcely conceivable that they are unstable and easily modified. Doubly improbable is Loew's hypothesis of the existence of special "living" proteins in which the amino groups are replaced by cyanide groups and in which aldehyde properties are still present. All this is said to bestow an unusual activity and instability on the "living proteins."² If such substances actually existed, there would be no trace of them noticed in the mass of the protein preparations, on account of their instability. Moreover, as we have already seen, the notions of the instability of the protoplasmic proteins are to be regarded as superceded.

33. ANALYTICAL METHODS

The total nitrogen in plant material is usually determined by the Kjeldahl method³ which is used in different modifications. The principle of this method is to burn the substance by boiling it with fuming sulphuric acid in the presence of various catalysts, whereupon all the nitrogen is converted to ammonia. After the addition of caustic soda in excess, the ammonia is distilled off into a titrated acid (HCl is best) and determined quantitatively by titration. Because the method is rapid and allows for the handling of several analyses at the same time, it is employed in the most varied kinds of investigations with the exception of analyses of unknown substances. In these cases, the analyses are carried out by the accurate and approved method of Dumas.⁴ The Kjeldahl method fails in analyses of substances with chains of nitrogen atoms (hydrazines and diazo compounds) and with oxidized nitrogen in the molecule, as in cyanide derivatives. Of all these substances, only nitrates can occur in the plant in appreciable amounts; to determine the nitrates, a Kjeldahl analysis as modified by Jodlbauer⁵ is useful. A solution of phenol is first added to the sulphuric acid to change the nitrates to nitro compounds. The nitro groups are then reduced with zinc or sodium hyposulphite and the Kjeldahl combustion can then take place without loss of nitrogen.⁶ The methods for the

¹ Wundt, W. *System der Philosophie*. 2: 84. 1907.

² Loew, O. *Science*. 11: 930. 1900 and the papers of the author which are cited there.

³ Kjeldahl, J. *Zeitschr. f. analyt. Chem.* 22: 366. 1883; Rona, P. *Handbuch. d. biochem. Arbeitsmethoden* by Abderhalden. 1: 340. 1910.

⁴ This method is described in all textbooks of organic chemistry.

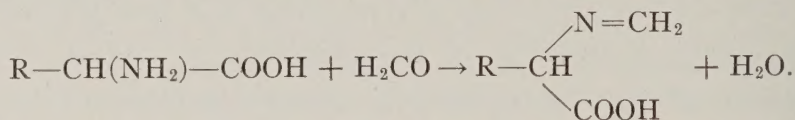
⁵ Jodlbauer, M. *Zeitschr. f. analyt. Chem.* 26: 92. 1887; Süllwald. *Chem. Zeit.* 11: 1673. 1903; Rona, P. *loc. cit.*

⁶ Some substances, such as purine nuclei, histidine, lysine, creatine, etc. are consumed only slowly and with difficulty by the Kjeldahl method. On this point see Kutscher, F. und Steudel. *Zeitschr. f. physiol. Chem.* 39: 12. 1903; Sörensen und Pedersen. *Ibid.*, p. 513; Schöndorff. *Pflügers Arch. f. ges. Physiol.* 98: 130. 1903; and others.

separation and purification of various physiologically important nitrogenous products have already been described (p. 286 ff.). The nitrogenous substances for the most part are not isolated in investigations in agricultural chemistry and physiology. It suffices to make quantitative measurements of the nitrogen in definite groups or compounds which are precipitated by definite reagents. The following quantitative methods are commonly used.

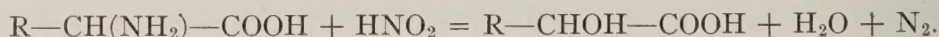
I. *Determination of protein nitrogen according to Stutzer.*¹ The liquid to be studied is treated with cupric hydroxide in the presence of alum or with copper sulphate in the presence of alkalis.² This precipitates the true proteins (not the peptones). The nitrogen in the precipitate is determined by the Kjeldahl method. The results obtained by Kostychev and Brilliant³ put the reliability of this method open to question. It was pointed out above that some determinations of "protein nitrogen" by the Stutzer method are to be regarded as erroneous, because copper hydroxide precipitates not only the protein nitrogen but also the nitrogen of compounds of amino acids and sugar. For the determination of protein nitrogen, we must find another method which involves no great technical difficulties.

II. *The determination of nitrogen of the amino groups* is done by two methods. Sørensen's method⁴ is as follows. Aldehydes react with the amino groups and a molecule of water is lost just as in the action of phenylhydrazine. The carboxylic acids are thereby formed from amino acids. For example, the following reaction takes place with formaldehyde:



The amphoteric nature of the amino acids disappears and the solution becomes acid. The free carboxyl groups are determined by titration and from this the number of amino groups are calculated. At this point the neutral solution of amino acids is treated with an exactly neutral solution of formaldehyde and the resultant acids are titrated with phenolphthalein. The inaccuracy of the method lies in the assumption that there is exactly one carboxyl group for every amino group. This ratio does not hold in the dibasic amino acids (aspartic and glutamic acid) or in the diamino acids. Moreover, the "formol method" is limited to colorless or only slightly colored solutions because dark-colored solutions cannot be titrated [with indicators]. Sørensen's scheme for decolorizing the experimental solution does not always give satisfactory results, according to the experience of the writer of this book.

Another method of determining the amino groups⁵ is based on the appearance of the nitrogen of primary amines in the molecular condition when they are treated with nitrous acid:



The reaction does not go on entirely smoothly because nitric oxide is also formed, but if the latter is decomposed with potassium permanganate, all the nitrogen changes to N₂. The pure nitrogen is measured in an azotometer, corrections are made for temperature and pressure, and the resulting figure in cubic centimeters is converted into milligrams of nitrogen. A source of error in this method is the appearance of nitrogen from ammonia which is often present in the material to be analyzed: $\text{NH}_4\text{NO}_2 = \text{N}_2 + 2\text{H}_2\text{O}$. Hence it is suggested that the ammonia be first removed by distilling under reduced pressure in the presence of lime, magnesia or soda and common salt.

¹ Stutzer. Jour. f. Landwirtschaft. 29: 473. 1881; König. Untersuchung landwirtschaftlich und gewerblich wichtiger Stoffe, p. 212. 1891.

² Barnstein. Landwirt. Versuchs-Stationen. 54: 327. 1900.

³ Kostytschew, S. und W. Brilliant. Zeitsch. f. physiol. Chem. 91: 372. 1914; Idem. Mitt. russ. Akad. d. Wiss., p. 953. 1916 (Russian); Idem. Jour. russ. bot. Ges. 5: 71 and 78. 1921 (Russian); Idem. Zeitschr. f. physiol. Chem. 127: 224. 1923; 130: 34. 1923.

⁴ Sørensen, S. P. L. Biochem. Zeitschr. 7: 43. 1907; Jessen-Hansen. Handbuch. d. biochem. Arbeitsmethoden. 6: 262. 1912.

⁵ van Slyke, D. D. Jour. Biol. Chem. 9: 185. 1911; Idem. Handbuch d. biochem. Arbeitsmethoden by Abderhalden. 5: 995. 1912; 6: 278. 1912.

III. *The determination of nitrogen of the amide groups* (CONH_2) gives an idea of the amount of asparagine and glutamine (and of urea) in the plant material to be studied. Unlike the amine groups, the amide groups are easily saponified by acids and alkalis. Sachsse's¹ method of determining the amide nitrogen is based on this point. The substance is extracted with hot water, the extract precipitated with tannic acid, and boiled for 2 hours in a reflux condenser after adding 10 cc. of concentrated hydrochloric acid to every 100 cc. of liquid. After cooling the liquid, it is neutralized and used for a determination of ammonia (see below). Ammonia is measured in an equal volume of liquid without previous boiling with HCl. The difference between the two determinations gives the amount of amide nitrogen. To calculate the total N of the asparagine and glutamine, this figure must be doubled because for every amide group they contain an amine group which is not removed by hydrolysis.

IV. *The determination of ammonia nitrogen* is best carried out by distilling the liquid under reduced pressure (10–15 mm.) and a temperature not higher than 40° in the presence of calcined magnesia or lime.² If the liquid contains dissolved phosphates or suspensions of solid particles, it is not advisable to use magnesia since it does not penetrate the tissue and forms the insoluble double ammonium salt of phosphoric acid. Since the wide distribution of urea in plants has been proved, it is inadmissible to distill the ammonia at 100° and atmospheric pressure. Under these conditions and with the alkaline reaction of the medium, the ammonia would be removed from the amide groups. It would be inaccurate to precipitate the ammonia with phospho-tungstic acid because this precipitation is never complete.³ Determination of ammonia by passing a rapid current of air through an alkaline solution of NH_3 ⁴ is somewhat bothersome and requires powerful water-jet vacuum pumps, but it gives exact results when all the necessary conditions are met.

V. *The determination of nitrogen of the diamino acids* is done separately by careful analytical work for arginine, lysine and histidine, since Kossel's method makes it possible to isolate these substances quantitatively and to separate them from one another (p. 287). For less detailed investigations it is sufficient to determine the nitrogen in the precipitate by phospho-tungstic acid by the Kjeldahl method, after the proteins and peptones have been removed by lead acetate and the excess of lead by hydrogen sulphide. The precipitate should be decomposed and the ammonia removed previous to the determination of nitrogen because the ammonia is precipitated by the phospho-tungstic acid.

VI. *The determination of the nitrogen of purine bases* is undertaken as follows.⁵ The nucleins are hydrolyzed in a reflux condenser for 6–10 hours at 100° ; very dilute (1–4%) sulphuric or hydrochloric acid is used for the hydrolysis. After neutralization and evaporation, the mineral acids are removed with barium chloride or silver nitrate, the purine bases precipitated with 10% ammoniacal silver solution and the nitrogen in the precipitate determined by the Kjeldahl method. This method is not entirely accurate. To get an idea of the amount of nuclein in the material to be studied, the nuclein phosphorus can also be determined by Plimmer's method.⁶ The protein precipitate is digested for 1–2 days at 37° with 1% caustic soda or gastric juice, the nucleoproteids precipitated with hydrochloric or acetic acid, the precipitate extracted with alcohol and ether after removing the phosphatides (see next chapter), and the phosphorus in the residue is determined by Neumann's method (p. 230 ff.).

¹ Sachsse. *Die Chemie und Physiologie der Farbstoffe, Kohlenhydrate, etc.* 1877; *Idem.* *Jour. f. prakt. Chem.* (2) 6: 118. 1873.

² Boussingault. *Ann. de chim. et de physique* (3) 29: 472. 1850; cf. also Krüger, M. und Reich. *Zeitschr. f. physiol. Chem.* 39: 165. 1903; Schittenhelm. *Ibid.*, p. 73; Grafe, E. *Ibid.* 48: 300. 1906; and others.

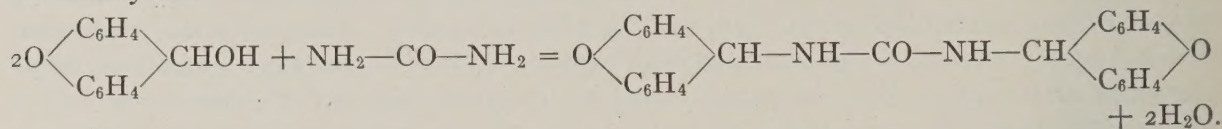
³ Bosshard, E. *Zeitschr. f. analyt. Chem.* 22: 329. 1883; Schulze, E. und Winterstein. *Ibid.* 33: 566. 1901; cf. especially Castoro, N. *Ibid.* 50: 525. 1907.

⁴ Folin. *Zeitschr. f. physiol. Chem.* 37: 161. 1902.

⁵ Burian und Schur. *Zeitschr. f. physiol. Chem.* 23: 53. 1897; Burian und Hall. *Ibid.* 39: 336. 1903; Krüger und Schittenhelm. *Ibid.* 45: 14. 1905; Krüger und Schmid. *Ibid.* 45: 1. 1905; Schittenhelm. *Handbuch d. biochem. Arbeitsmethoden* by Abderhalden. 3: 884. 1910; Zaleski, W. *The transformations and the rôle of phosphorus compounds in plants.* 1912 (Russian).

⁶ Plimmer and Bayliss. *Jour. Physiol.* 33: 439. 1906; Plimmer and Scott. *Ibid.* 38: 247. 1909; Zaleski. *loc. cit.*

VII. *The determination of urea* is done best by means of the exact and delicate method of Fosse:¹ in acetic acid solution urea yields an insoluble crystalline compound with two molecules of xanthydrol:



The molecular weight of urea is 60 and that of dixanthyl urea is 420; hence 0.0001 g. of urea can be detected microchemically because dixanthyl urea crystallizes so well. By this method urea can be determined in an extremely dilute solution (1 part in a million). The precipitate is either weighed or used for a nitrogen determination.

¹ Fosse, R. Compt. rend. 154: 1187. 1912; 157: 948. 1913; *Idem.* Ann. Inst. Pasteur. 30: 1. 1916.

CHAPTER VII

SECONDARY PLANT SUBSTANCES

1. THE MODERN PROBLEMS OF PLANT CHEMISTRY

The carbohydrates and proteins are the primary building materials of the seed-plants; they are formed from inorganic substances in the light and by transformations furnish all the organic compounds required by the plants. In the preceding chapters it was explained that the plants without chlorophyll also first synthesize carbohydrates and proteins from their nutrient substrate. Thus it is advisable to distinguish between primary and secondary substances.

The secondary substances include those which are not generally distributed; on the contrary, most of them are characteristic of certain groups of plants. Of course, this makes it difficult to estimate correctly the physiological rôles of the individual secondary plant substances, because it is very probable that the same physiological function is fulfilled in different plants by substances of unlike composition. Consequently the clarification of the physiological rôles of the various secondary substances is in many cases a premature problem, the more so because many purely chemical questions are still unsettled, the solution of which must take precedence over all teleological explanations. In some manuals of plant chemistry and plant physiology one finds a classification into "Substances of intermediate metabolism," which are therefore subject to further change in the living cells, and the so-called "End-products of metabolism," or simply waste materials which are said to have no further use. This classification is often arbitrary and even erroneous because different substances once thought to be end-products are now shown to be intermediate products of metabolism and enzymes have been discovered, which work on these products. In general, it is very hard to prove that a given substance is not used further.

Hence, the modern problems of plant chemistry do not refer to the explanation of the "physiological rôles" of the various substances but to the following real questions. Concerning substances of unknown chemical structure, it must first be determined to what substances of known chemical nature they are related, so the following questions must first be solved: 1. From what previous substance, protein or sugar, is the unknown substance derived? 2. Which chemical change from sugar or protein leads to the formation of the given secondary substance? 3. If the substance in question is used further, the chemical nature of this transformation must also be studied. Both the first and third questions could be solved empirically in many cases. The genetic connection between sugar and fat was established in this way. As for the second question, it belongs in the category of the chemical problems, the experimental attack on which must be based on a working hypothesis. The method of

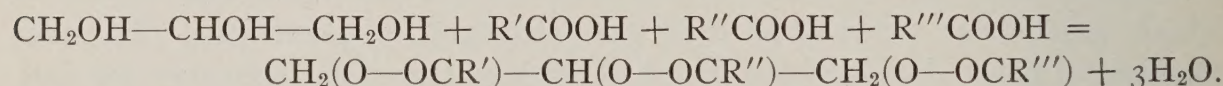
isolation of the intermediate products of the given transformations is often the only means of learning the chemical nature of biochemical processes which are composed of several coupled reactions. Since the methods of isolation of the intermediate products of the biochemical reactions may be very varied (*cf.* p. 62), it is essential to have a preliminary hypothetical idea of the intermediate products to be expected.

Since all the chemical problems discussed above are still entirely new, one finds in the current literature few experimental investigations which bear on the given problems: this fruitful work awaits future investigation for the most part. Consequently this chapter devotes special attention to the various hypotheses of the methods of formation of the secondary substances. Such hypotheses will be needed in the coming experimental investigations. After the purely chemical questions are disposed of, it will be possible to judge correctly the physiological rôles of the individual secondary substances.

There are excellent manuals of plant chemistry in which complete information may be found for all the more important plant substances. Hence, we shall mention only some of the most important substances in order to give by these examples the fundamental considerations of the chemistry of the methods of formation of the various substances in plants.

2. FATS

Glycerine is a trivalent alcohol and can therefore unite with three molecules of different acids:



Such glycerine esters or glycerides form the physiologically very important group of fats.¹ The variability of the fats is due first to the acids in the glyceride molecule, secondly to combinations of different glycerides, and thirdly to the possible presence of free acids in the fat complex.² The acids in a glyceride molecule are not always the same. Among those present in glycerides, the following are especially widespread:

Palmitic acid $\text{CH}_3-(\text{CH}_2)_{14}-\text{COOH}$

Stearic acid $\text{CH}_3-(\text{CH}_2)_{16}-\text{COOH}$

Oleic acid $\text{CH}_3-(\text{CH}_2)_7-\text{CH}=\text{CH}-(\text{CH}_2)_7-\text{COOH}$

Linoleic acid $\text{CH}_3-(\text{CH}_2)_4-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}=\text{CH}-(\text{CH}_2)_7-\text{COOH}$, and

Linolenic acid $\text{CH}_3-\text{CH}_2-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}=\text{CH}-$
 $(\text{CH}_2)_7-\text{COOH}.$

In addition to these, other acids have been found in fats, including the following saturated acids:

Acetic acid CH_3-COOH

Propionic acid $\text{CH}_3-\text{CH}_2-\text{COOH}$

¹ Chevreul. *Ann. de chim.* (1) 88: 225. 1815; *Idem.* *Ann. de chim. et de physique* (2) 2: 339. 1816; 7: 155. 1817; 16: 197. 1821; 23: 16. 1823; and others. These pioneer papers were the first steps in the field of biochemistry.

² On the regularities in the glyceride structure of fats found in plants, see Collin and Hilditch. *Biochem. Jour.* 23: 1272-1289. 1929.—*Ed.*

Normal butyric acid $\text{CH}_3\text{—CH}_2\text{—CH}_2\text{—COOH}$

Iso-butyric acid $\text{CH}_3\text{—CH}(\text{CH}_3)\text{—COOH}$

Caprylic acid $\text{CH}_3\text{—}(\text{CH}_2)_6\text{—COOH}$

Lauric acid $\text{CH}_3\text{—}(\text{CH}_2)_{10}\text{—COOH}$

Myristic acid $\text{CH}_3\text{—}(\text{CH}_2)_{12}\text{—COOH}$, and others.

Among the hydroxy acids found there is

Ricinoleic acid $\text{CH}_3\text{—}(\text{CH}_2)_5\text{—CHOH—CH}_2\text{—CH=CH—}(\text{CH}_2)_7\text{—COOH}$ and the isomeric dihydroxy stearic acid.

The unsaturated fatty acids are also rather numerous. The high percentage of unsaturated fatty acids is a mark of the plant fats because chiefly saturated fatty acids are present in animal fats. Tropical plants also contain considerable amounts of glycerides of saturated acids.^a

The glycerides of unsaturated acids are liquids at ordinary temperatures. There are more acids with a double bond in drying oils which are very important in the preparation of dyes and lacquers. The drying depends partly on the formation of saturated acids and partly on the formation of oxyacids.¹ Hence, the drying is facilitated by all agencies which increase the velocity of the oxidation processes.

Fats are insoluble in water and cold alcohol but are very soluble in ether, carbon disulphide, petrol ether, benzene and boiling alcohol. An isolation of chemically pure glycerides from natural products involves enormous difficulties and the physical constants of the oils are not sharply defined because the natural fats are only complex mixtures of various glycerides and free fatty acids. Acids and alkalis effect hydrolysis or the so-called saponification of fats, even at low temperatures. At high temperatures they saponify very rapidly. The saponification products are glycerine and soaps, *i.e.*, salts of fatty acids, if the catalyst is an alkali. In plant cells the saponification is effected by the enzyme lipase.² An acid reaction favors the action of lipase so that physiological saponification of fats is a typical case of so-called autocatalysis.

In the presence of a small amount of mineral acids, 1 gram of powdered *Ricinus* seeds decomposes 25 g. of fat in a couple of days.³ A reversal of the action of lipase is often observed⁴ and glycerides can be synthesized with certainty under the influence of lipase.^b

^a Correlations of the structure of fats and related compounds with climatic and taxonomic factors of a wide variety of plants have recently been brought out by **McNair**. *Amer. Jour. Bot.* **16**: 832–841. 1929. —*Ed.*

¹ **Bauer und Hazura**. *Monatsch. d. Chem.* **9**: 459: 1888.

² **Green**. *Ann. Bot.* Vol. **4**: 1890; **Idem**. *Proc. Roy. Soc. London.* **47**: 146. 1891; **48**: 370. 1891; **Green and Jackson**. *Ibid.* (B) **77**: 69. 1905; **Willstätter, R. und Waldschmidt-Leitz**. *Zeitschr. f. physiol. Chem.* **134**: 161. 1924.

³ **Connstein**. *Ber. chem. Ges.* **35**: 3988. 1902; **Idem**. *Ergebn. Physiol.* **3** (I): 194. 1904; **Hoyer**. *Ber. chem. Ges.* **37**: 1436. 1904; **Idem**. *Zeitschr. f. physiol. Chem.* **50**: 414. 1907; and others.

⁴ Among others on this point *cf.* **Bradley, H. C.** *Jour. Biol. Chem.* **8**: 251. 1910; **Welter, A.** *Zeitschr. f. angew. Chem.* **24**: 385. 1911; **Iwanoff, S. L.** The formation and transformation of fat in seed-plants. 1912 (Russian); **Dunlop and Gilbert**. *Jour. Amer. Chem. Soc.* **33**: 1787. 1911; **Krausz, M.** *Zeitschr. f. angew. Chem.* **24**: 829. 1911; **Spiegel, L.** *Zeitschr. f. physiol. Chem.* **120**: 102. 1922.

^b This action is so positive and dependable that it forms an excellent experiment for classes in physiology, biochemistry, etc. in connection with either fats or enzymes. A mixture of almost any fatty acid, a little water and an active preparation of a lipase may be made at one meeting of the class, the acidity determined by titration of a sample, and the container left closed until the next meeting of the class when enzyme action can be demonstrated by a second titration which will show a lower acid content in the mixture.—*Ed.*

In plants the fats are reserve substances which are used to form carbohydrates and for respiration. The advantages of fats as reserve material are as follows. In the first place, they are insoluble in water; hence they cannot enter into reactions in aqueous solution before being saponified. Secondly, it is important that the fluid droplets of fat are easily forced into any shape and so they occupy less space than solid particles which always have empty spaces between them. Thirdly, the fats have a high heat of combustion which is a great advantage in respiration. The following table gives the heats of combustion of different fats and of other physiologically important substances.¹

1 gram of substance	Yields heat in calories
Linseed oil.....	9323
Olive oil.....	9328
Poppy-seed oil.....	9442
Stearic acid.....	9429
Glycerine.....	4317
Grape sugar.....	3692
Starch.....	4123
Protein.....	5567

We are therefore justified in calling oil a very good reserve substance. It is very widely distributed in seeds, cortical parenchyma and other reservoirs of reserve foods. Fats are found as nitrogen-free reserve materials in about four-fifths of the phanerogams.² In the *Cruciferae* the fat content reaches 60% (*Lepidium*), in the *Linaceae* 40%, in the *Compositae* 50%, in the *Euphorbiaceae* 70% (*Ricinus*), etc.

The fats are consumed during seed germination, as the following table³ shows:

	Fat in grams
<i>Raphanus sativus</i>	
5 g. of ungerminated seeds.....	1.750
5 g. of 2-day old seedlings.....	1.635
5 g. of 3-day old seedlings.....	1.535
5 g. of 4-day old seedlings.....	0.790
<i>Papaver somniferum</i>	
20 g. of ungerminated seeds.....	8.915
20 g. of 2-day old seedlings.....	6.815
20 g. of 4-day old seedlings.....	3.900

Fat can be used only after saponification. The glycerine is changed so fast that generally it cannot be detected. The fatty acids remain for some time in the free condition but they are finally consumed, the plant using the

¹ Stohmann, F. Jour. f. prakt. Chem. 19: 115. 1879; 31: 273. 1885; Idem. Zeitschr. f. Biol. 13: 364. 1894.

² Nägeli. Stärkeköerner, p. 467. 1858.

³ Boussingault. Agronomie, chimie agric. et physiol. 5: 50. 1874.

unsaturated acids first.¹ With a shortage of oxygen, glycerine can be seen to form because the process which uses it is thereby retarded.² Analogous conversions of fat can be observed in the bark of various kinds of trees in the spring.³ However the older statements concerning the conversion of reserve starch into fat⁴ must be corrected:⁵ both starch and fat appear to accumulate in the bark at the same time but it is very difficult to detect fat in the cells of the bark parenchyma microscopically before there has been sufficient conversion of starch.

The work of S. L. Iwanoff⁶ furnishes interesting observations concerning the formation of fat in various plants. The heat of combustion of stored fats is greater in the northern than in the southern plants, due to the content of unsaturated acids which is lower in the tropical plants than in those of northern regions. Moreover, it seems that entirely new fatty acids have appeared during the evolution of the plant kingdom. For example, linolenic acid is lacking in all the lower plants. Finally it is noteworthy that the same fats are produced by the newly-formed species of plants. This rule also applies for terpenes, glucosides, alkaloids and a few other substances. For example, in connection with the fats, it is possible to predict the stored substances in new species which have never been investigated.⁷ S. L. Iwanoff believes that plant evolution as a rule is due to a change in the physiological transformations of substances, and that the newly-formed substances characterize the course of evolution. According to Iwanoff (*loc. cit.*), this is the way that different fats, alkaloids, indol derivatives and many other substances appeared at certain periods in the evolution of plants.

It is easy to follow the formation and accumulation of different fats during the ripening of fatty seeds and fruits. Fat appears to be very seldom formed in leaves, since the supposed droplets of fat which the older workers observed in the chloroplasts of *Vaucheria*, *Musa* and a few other plants were later shown to be composed of other substances (*cf.* p. 110 and 112). In ripening olives the fat content increases in the following manner:⁸

At the end of June.....	1.40 per cent
At the end of July.....	5.49 per cent
At the end of August.....	29.19 per cent
At the end of September.....	62.30 per cent
At the end of October.....	67.21 per cent
At the end of November.....	68.57 per cent

¹ Muntz. Ann. de chim. et de physique (4) 22: 472. 1871; Leclerc du Sablon. Compt. rend. 117: 524. 1893; 119: 610. 1894; Idem. Rev. gén. bot. 9: 313. 1897; Green and Jackson. Proc. Roy. Soc. London (B) 77: 69. 1905; Iwanoff, S. L. Jahrb. wiss. Bot. 59: 375. 1912.

² Grafe, V. und O. Richter. Sitzungsber. d. Akad. Wien, Math. natur. Kl. 1911.

³ Iwanoff, S. L. Mitt. d. Bur. f. spez. Pflanzenzucht. 4: 1. 1917 (Russian).

⁴ Russow. Sitzungsber. d. naturforsch. Ges. d. Univ. Dorpat, p. 402. 1882; Fischer, A. Jahrb. wiss. Bot. 22: 73. 1890.

⁵ Niklewski. Beih. bot. Centralbl. 19 (I): 68. 1905; *cf.* also Vandevelde. Chem. Centralbl. (I), p. 466. 1898.

⁶ Iwanoff, S. L. The formation and transformation of fat in seed-plants. 1912 (Russian); Idem. Mitt. d. Bur. f. spez. Pflanzenzucht. Nos. 1 and 7. 1905; Nos. 2 and 3. 1914; No. 7. 1915; Nos. 7 and 12. 1916; No. 1. 1917 (Russian); Idem. Beih. bot. Centralbl. 28 (1): 159. 1912; Idem. Jahrb. wiss. Bot. 50: 375. 1912; Idem. Fortschritte d. naturwiss. Forsch. New Series, part 3. 1929; Pigulewski. Jour. russ. chem. Ges. 48: 324. 1916 (Russian).

⁷ Iwanoff, S. L. *loc. cit.*

⁸ Rousille. Compt. rend. 86: 610. 1878; *cf.* also the thorough investigations of Scurti and Tommasi. Ann. staz. agric. sperim. di Roma (2) 4: 253. 1910; 5: 103. 1912; 6: 29. 1913.

In this case also there is first an accumulation of free acids,¹ followed by a synthesis of the glycerides. From quantitative determinations of the double bonds it is evident that the saturated acids are formed first and that the unsaturated acids are formed from them.² There is always a corresponding consumption of sugar when fat is formed.³ The nature of the respiratory metabolism also indicates that fats are produced from sugar. At the time when fat begins to form during the ripening of seeds, the ratio $\frac{\text{CO}_2}{\text{O}_2}$ (the molecular ratio of the carbon dioxide released to the oxygen absorbed) is considerably larger than 1. This shows that part of the oxygen from the sugar is liberated in the form of carbon dioxide, in addition to the oxygen taken in.⁴ The formation of fat from sugar is actually a reduction process; since carbohydrates contain more oxygen than fatty acids, the excess oxygen is released in the form of carbon dioxide. The opposite process of resorption of fat during seed germination involves the formation of sugar.⁵ The same process can be seen in the bark of trees in the spring.⁶ In one single experiment of this kind, the bark contained 0.004% grape sugar and 3.4% fat. After 3 days at room temperature the content of grape sugar has increased to 0.58% and after 6 days to 1.64%; in the meantime the fat content fell to 2.0%. When fat is being consumed, the value of $\frac{\text{CO}_2}{\text{O}_2}$ is always considerably less than 1, because the surplus of oxygen in the gas exchange is used to synthesize sugar from fats.

The conversion of fat in the seed-plants has already been considered. Analogous processes are observed in the lower plants but there have not been many experimental studies in this field. Careful experiments on the use of fat by the lower fungi show that the economic coefficient (*cf.* p. 182) is very large in this case, corresponding to the enormous heat of combustion of fat.⁷ The fat content of the tubercle bacteria reaches 40% of the dry weight and a few other bacteria also have a high content of fat. The general transformations of fat in bacteria seem to be identical with those in seed-plants.⁸ Lipase is also found in bacteria.⁹ In yeast cells the fat content likewise becomes high under certain conditions.¹⁰ However, it must be borne in mind that in alcoholic fermentation most of the glycerine, contrary to the statements of some earlier workers, comes not from fat but from the sugar (*cf.* Chap. IX).¹¹

¹ Leclerc du Sablon. *Compt. rend.* 123: 1084. 1896.

² Iwanoff, S. L. The formation and transformation of fat in seed-plants. 1912 (Russian).

³ Leclerc du Sablon. *loc. cit.*

⁴ Gerber, C. *Compt. rend.* 135: 658 and 732. 1897.

⁵ Leclerc du Sablon. *Compt. rend.* 117: 524. 1893; 119: 610. 1894; Maquenne, L. *Ibid.* 127: 625. 1908.

⁶ Iwanoff, S. L. *Mitt. d. Bur. spez. Pflanzenzucht.* Vol. 4, no. 1. 1917 (Russian).

⁷ Flieg, O. *Jahrb. wiss. Bot.* 61: 24. 1922.

⁸ Kressling. *Centralbl. f. Bakt. (I)* 30: 897. 1901; and others.

⁹ Söhngen. *Kgl. Akad. Amsterdam.* 19: 667 and 1263. 1910; and others.

¹⁰ Nadson, G. *Tageb. d. 1 Kongr. russ. Bot.*, p. 102. 1921 (Russian).

¹¹ Lindner, P. *Zeitschr. f. angew. Chem.* 35: 110. 1922 assumes that yeast forms glycerine from alcohol by the intermediate stage of acetaldehyde. However, this conception is contrary to various pieces of experimental evidence which show that yeasts, like other plants, build their reserve fats from sugar. On this point see Smedley-Maclean, Ida. *Biochem. Jour.* 16: 370. 1922.

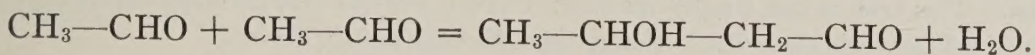
Fat is likewise a very common reserve substance in the higher fungi, algae and lichens. Unfortunately, the interesting problem of the transformations of fat in the higher fungi and algae has not been solved. There is no doubt of the presence of lipase in the fungi.¹ As for the Pteridophytes, our knowledge of the handling of fat in these plants is entirely insufficient.

The genetic connection between fat and sugar can therefore be regarded as established by numerous experiments. There are plenty of cases of conversion of fat to sugar and of the reverse process of forming fat from sugar. However, these processes are neither artificially feasible nor chemically understood. This is a great gap in plant chemistry which is in urgent need of being closed.

The problem of conversion of sugar to fat resolves into two different questions: the problem of forming glycerine and that of making the fatty acids. From the chemical standpoint, the formation of glycerine from sugar is quite conceivable. A cleavage of the hexose molecule into two molecules of a "three-carbon substance" is a part of almost all modern theories of alcoholic fermentation. The explanation of glycolysis in animal tissues is also based on this hypothesis. Glycerine can come by direct reduction or through conversion by the Cannizzaro reaction from the three-carbon substances in question. Investigations of the formation of sugar from glycerine by molds show that a substance with the properties of glyceric aldehyde seems to be formed by this process.²

The problem of forming the acids from sugars is less simple. Both reductions and synthetic processes must go on at the same time. E. Fischer believes that fatty acids with 18 carbon atoms are formed by the linkage of three glucose molecules, followed by reduction.³ The formation of other fatty acids is not explained by this theory. The other theory⁴ is more probable, according to which the fatty acids are synthesized from sugar by way of acetaldehyde as the intermediate step. This theory is now supported by the fact that acetaldehyde has actually been found as an intermediate product of alcoholic fermentation.⁵ Hence we may assume that acetaldehyde is formed in every cell which has the power of fermentation and this is generally widespread (see Chap. VIII). Acetaldehyde has actually been observed to appear during the formation of fat.⁶ The theory is therefore now well-founded.

According to v. Euler, fatty acids are synthesized as follows: two molecules of acetaldehyde first unite as an aldol:



¹ Camus. Bull. Soc. Biol., p. 192 and 230. 1897; Garnier. *Ibid.* 55: 1490. 1903; Gérard. Compt. rend. 124: 370. 1897.

² Kostytschew, S. Zeitschr. f. physiol. Chem. 111: 236. 1920.

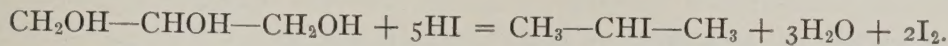
³ Fischer, E. Untersuchungen über Kohlenhydrate und Fermente. 1909.

⁴ Raper, H. S. Proc. Chem. Soc. 23: 235. 1907; Idem. Jour. Physiol. 32: 216. 1906; Euler, H. v. Pflanzenchemie. 3: 212. 1909; Franzen, H. Zeitschr. f. physiol. Chem. 112: 301. 1921; Haehn, H. Zeitschr. f. tech. Biol. 9: 217. 1921.

⁵ Kostytschew, S. Zeitschr. f. physiol. Chem. 79: 130. 1912; 83: 93. 1913; Kostytschew und Scheloumow. *Ibid.* 85: 493. 1913; Kostytschew und Brilliant. *Ibid.* 85: 507. 1913; Kostytschew, S. Biochem. Zeitschr. 64: 237. 1914; and others; cf. p. 427.

⁶ Haehn, H. und W. Kinttoff. Ber. chem. Ges. 56: 439. 1923; Idem. Chemie d. Zelle und Gewebe. 12: 115. 1925.

The best way to make quantitative determinations of glycerine is to find the number of methoxyl groups CH_3O .¹ Treatment with hydriodic acid changes glycerine to isopropyl iodide:



The resulting product is driven by a current of air into a solution of AgNO_3 and the number of methoxyl groups is calculated from the silver iodide that is formed.

The determination of fatty acids is based on their isolation and separation. Lead salts of the acids of the oleic series are soluble in ether. Isolation of the individual glycerides involves enormous difficulties and is usually not done. The following constants are entirely sufficient to characterize the individual fats.

1. *The saponification number* or the mg. of KOH required to saponify 1 g. of fat.²
2. *The iodine number* or the per cent of iodine taken up by the fat.³ This constant indicates the number of double bonds because iodine or bromine takes the place of the double bond. The solution of fat in chloroform is added to a standardized alcoholic solution of iodine containing mercuric chloride. After standing for some time, the excess iodine is titrated with thiosulphate.

3. *The percentage of free acids* is measured by direct titration.

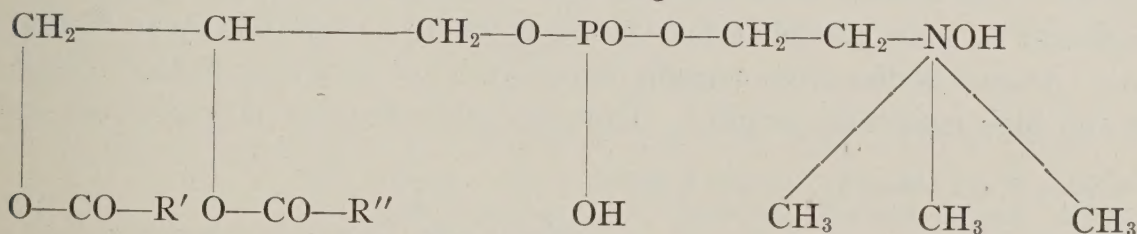
4. *The Reichert-Meissl number* or the number of cubic centimeters of 0.1 N NaOH required to neutralize the liquid fatty acids distilled off from 5 g. of fat.

In addition one often determines the percentage of unsaponifiable substances that are soluble in ether,⁴ and the insoluble fatty acids.

All determinations of fats are carried out with an ether extract of the material to be investigated. Extractions with petrol ether have been preferred in recent years because less foreign substances are removed and because the petrol ether extract can be purified considerably by shaking with water.^d

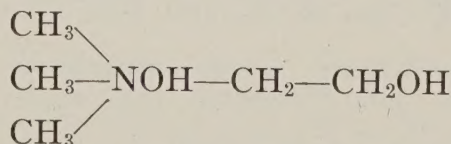
3. LECITHINS AND PHOSPHATIDES

Lecithins are fats of the following structure which contain nitrogen and phosphorus: only two hydroxyl groups of the glycerine are replaced by fatty acids; the third hydroxyl group is linked like an ester with ortho-phosphoric acid and the latter is itself united in the same way with choline. The general formula for lecithin is therefore the following:⁵



in which $\text{R}'-\text{COO}$ and $\text{R}''-\text{COO}$ represent different fatty acid residues.

Choline:



¹ Zeisel und Fanto. Monatsch. f. Chem. 6: 989. 1885; Stritar. Zeitschr. f. analyt. Chem. 42: 579.

² Köttstorfer. Zeitschr. f. analyt. Chem. 18: 199. 1879.

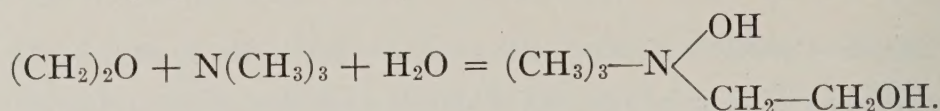
³ Walter. Chem. Zeit., p. 1786 and 1831. 1895.

⁴ Hönig, M. und Spitz. Zeitschr. f. analyt. Chem. 31: 477. 1892.

^d An excellent review of methods for estimating fats and fat-like substances in plant tissues is given by Sando. Plant Physiol. 3: 155-184. 1928. The term "lipides" is taken to cover fats and "lipoids" and all these substances are treated together.—Ed.

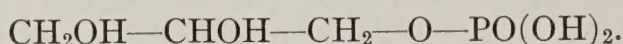
⁵ Diakonow. Med.-chem. Untersuchungen, p. 221. 1867 and p. 405. 1868; Strecker. Liebigs Ann. d. Chem. 148: 77. 1868.

can be easily removed from the lecithins.¹ The structure of this substance has been confirmed by synthesis from trimethyl amine and ethylene oxide:²

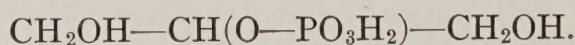


Choline is an alcohol and at the same time a strong base. It is very soluble in water and alcohol but only slightly soluble in ether. It is decomposed by boiling in aqueous solution.

If both choline and the fatty acids are removed from the lecithin molecule, there remains the optically active glycerophosphoric acid:³



Phosphoric acid is therefore linked with a primary alcohol group of the glycerine; a linkage of the phosphoric acid with the secondary group would give an optically inactive acid:



In this way the structural formula for the lecithins has been proved for its fundamental nature but not in all details. The glycerophosphoric acid is a sirupy substance which yields crystalline stable salts of calcium and barium.

The variations in the individual lecithins are due primarily to the variations in the acid residues R' and R''. Plants have been found to contain lecithins which are apparently closely related to the lecithin of egg yolk.⁴ Choline in the free condition has also been isolated from plants.⁵ Lipase can only remove fatty acids from lecithin as it does from the simple fats. Only a specific enzyme, the so-called glycerophosphatase, decomposes glycerophosphoric acid into glycerine and phosphoric acid.⁶

The complicated phosphatides are to be differentiated from the simple lecithins. The former often contain carbohydrate groups and other organic complexes:⁷ in some phosphatides, choline seems to be replaced by an inorganic base.⁸ Animal bodies often contain phosphatides of very complicated composition and high molecular weight.⁹ The plant phosphatides have not been suffi-

¹ Schulze, E. und Likiernik. *Zeitschr. f. physiol. Chem.* 15: 413. 1891.

² Wurtz. *Ann. de chim. et de pharmacie.* 6: 116 and 197. 1868.

³ Ulpiani. *Gazz. chim. ital.* 31(II): 47. 1901; Willstätter, R. und Lüdecke. *Ber. chem. Ges.* 37: 3753. 1904.

⁴ Schulze, E. und Frankfurt. *Landwirt. Versuchs-Stationen.* 43: 307. 1894; Schulze und Merlis. *Ibid.* 48: 203. 1897; Schulze, E. *Ibid.* 67: 57. 1907; Idem. *Zeitschr. f. physiol. Chem.* 55: 338. 1908.

⁵ Schulze, E. *Zeitschr. f. physiol. Chem.* 12: 414. 1888; 15: 140. 1891; 17: 140 and 193. 1892; Schulze und Trier. *Ibid.* 81: 53. 1913; Winterstein. *Ibid.* 95: 310. 1915; Yoshimura. *Ibid.* 88: 334. 1913; and others.

⁶ Iwanoff, S. L. *loc. cit.*; Nemec, A. *Biochem. Zeitschr.* 93: 94. 1918; 138: 198. 1923.

⁷ Schulze, E. *Zeitschr. f. physiol. Chem.* 52: 54. 1907; 55: 338. 1908; Winterstein und Hiestand. *Ibid.* 47: 496. 1906; 54: 288. 1908; Hiestand. *Diss. Zurich.* 1906; Winterstein und Smolenski. *Zeitschr. f. physiol. Chem.* 58: 506. 1909; Smolenski, K. *Ibid.* 58: 522. 1909; Lüers. *Zeitschr. f. ges. Brauw.* 38: 97. 1905; and others. Trier. *Über einfache Basen.* 1912 considers that the carbohydrate groups are an accidental impurity.

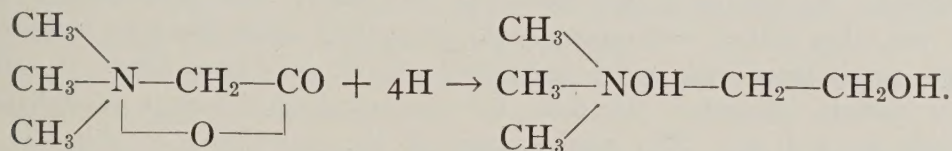
⁸ Burow. *Biochem. Zeitschr.* 25: 165. 1910; Glikin. *Ibid.* 21: 348. 1909; Stern und Thierfelder. *Zeitschr. f. physiol. Chem.* 53: 371. 1907.

⁹ On this point see Tudichum. *Chemische Konstitution des Gehirns.* 1901.

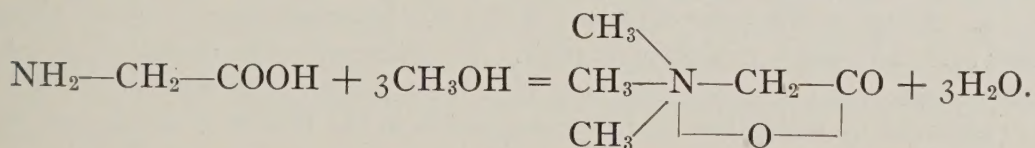
ciently investigated. It is not even certain that they all contain choline. E. Schulze¹ believes that in some plants choline is replaced by betaine or trigonellin. In one phosphatide a content of 6.7% calcium was found² and it may be assumed that in this case the organic base is replaced by calcium. Other investigators believe that phosphatides cannot exist without choline.³ Such contradictions can be disposed of only after improvement of the technique of isolating the phosphatides.

Lecithins and phosphatides are very widespread in the plant kingdom. They have also been found in plants without chlorophyll. Many writers are inclined to assume that lecithins, like the proteins, form the colloidal substrate of the protoplasmic framework and therefore occur in every living cell. Like the proteins, the lecithins are amphoteric electrolytes.

The phosphatides differ from the fats in that they occur only in small quantities in reservoirs of reserve materials. Only extremely small amounts of lecithin have been found in seeds⁴ but there are large quantities of phosphatides in young, growing tissues,⁵ rich in protoplasm, such as buds, pollen, etc. The phosphatides in seeds are split during germination⁶ and the nitrogen and the phosphorus of these compounds passes to the growing parts of the plant where new organs are being formed and the phosphatides are regenerated. Some writers⁷ have expressed the view that the phosphatides are first synthesized in illuminated leaves. This hypothesis is valid only with respect to choline because of course both fatty acids and glycerine are produced from sugar as explained in the consideration of the synthesis of fats. Choline may be formed from betaine by reduction:



Undoubtedly betaine is formed from glycocoll (p. 310):



It could also be assumed that phosphatides are synthesized partly from carbohydrates and partly from the products of protein cleavage. Yet Trier⁸

¹ Schulze, E. Landwirt. Versuchs-Stationen. 73: 89. 1910; Idem und Pfenninger. Zeitschr. f. physiol. Chem. 71: 74. 1911.

² Winterstein, E. und Stegmann. Zeitschr. f. physiol. Chem. 58: 500 and 527. 1909.

³ Trier. loc. cit.

⁴ Schulze, E. und Steiger. Zeitschr. f. physiol. Chem. 13: 365. 1889; Schulze, E. und Frankfurt. Landwirt. Versuchs-Stationen. 43: 307. 1899; Schulze, E. Ibid. 49: 203. 1899; 73: 89. 1910.

⁵ Stoklasa, J. Sitzungsber. Akad. Wien. 104: 617. 1896.

⁶ Schulze, E. und Steiger. loc. cit.; Schulze, E. Zeitschr. f. physiol. Chem. 17: 207. 1892; 40: 116. 1903; Prianschnikow. The decomposition of proteins during seed germination. 1895 (Russian); Zaleski, W. The transformation and rôle of phosphorus compounds in plants. 1912 (Russian).

⁷ Stoklasa, J. Zeitschr. f. physiol. Chem. 25: 398. 1898; Idem. Ber. chem. Ges. 29: 2761. 1896.

⁸ Trier. loc. cit.

disputes the hypothesis that betaine is the parent substance of choline and believes to have shown that both glycocoll and choline are formed at the same time during photosynthesis (p. 300).

The physiological rôle of the phosphatides has not yet been made clear although there are many hypotheses concerning it. Some investigators assume that the plasma membrane is composed largely of phosphatides.^e Moreover, the phosphatides have the remarkable property of bringing into solution various substances which are not very soluble.¹ The probable importance of phosphatides in the oxidation processes² and in photosynthesis³ has also been pointed out. Finally, the hypothesis is not precluded that phosphatides form the molecular complexes which are characteristic of living protoplasm. It has already been mentioned that the protoplasmic proteins are not held in the free condition but are united with other molecular complexes; in this way all the protoplasm of the living cell is one enormous chemical molecule, since all its constituents seem to be linked together. Phosphatides may be important in the process of forming complex molecules because they are reactive to a high degree.

In conclusion it must be emphasized that lecithins are formed in seed-plants by synthesis. The seed-plants are unable⁴ to absorb lecithin from its aqueous solutions through their roots.

Analytical Methods.—Like the fats, phosphatides are soluble in various organic solvents but least so in acetone. This is the basis of the preparation of phosphatides from plant material. The finely divided and dried material is extracted with acetone to remove the fats and phytosterins; the residue is then extracted with ether. The phosphatides are precipitated from the ether extract with acetone, the precipitate again dissolved in ether and the phosphatides precipitated with water containing a small amount of sulphuric acid, sodium sulphate or sodium chloride.⁵ Methods for separating the various phosphatides have not yet been worked out. The measurement of the phosphorus in ether or alcoholic extracts is used for quantitative determinations of phosphatides. For the latter it is inadmissible to use a preliminary treatment with acetone because part of the phosphatides go into the acetone. However, this is not necessary in this case because neither inorganic phosphates nor nucleic acids are soluble in alcohol or ether. The material is first extracted for 5–6 hours in a Soxhlet apparatus with pure ether, and then for 2–3 hours with absolute alcohol. The solvent is removed by distillation and the phosphorus in the residue is determined by Neumann's method.^f

^e On the other hand, there are some indications that they are not present in appreciable amounts in this membrane; cf. Steward, F. C. *Biochem. Jour.* 22: 268–275. 1928.—Ed.

¹ Winterstein, E. und Stegmann. *loc. cit.*; Michaelis und Rona. *Biochem. Zeitschr.* 2: 219. 1906; 3: 109. 1906; 4: 11. 1907; Reiss. *Hofmeisters Beitr. z. chem. Physiol. und Path.* 7: 151. 1905; and others.

² Palladin, W. *Ber. bot. Ges.* 28: 120. 1910; Palladin und Stanewitsch. *Biochem. Zeitschr.* 26: 351. 1910; Korsakowa, M. *Ibid.* 28: 121. 1910.

³ Ostwald, Wo. *Kolloid-Zeitschr.* 33: 356. 1923.

⁴ Schulow, I. *Investigations in the field of the physiology of nutrition of the higher plants*, p. 157. 1913 (Russian).

⁵ Schulze, E. *Zeitschr. f. physiol. Chem.* 55: 338. 1906; Idem. *Landwirt. Versuchs-Stationen.* 73: 89. 1910; Zaleski, W. *The transformation and rôle of the phosphorus compounds in plants.* 1912 (Russian).

^f For the detection of choline in plants see Klein and Zeller. *Oesterr. Bot. Zeitschr.* 79: 40–57. 1930.—Ed.

4. PHYTOSTERINS

Along with fats and phosphatides, ether and boiling alcohol extracts of plant material also contain substances which resemble the phosphatides in their physical properties but are very unlike them chemically. These are the so-called phytosterins which resemble the cholestrin of the liver. The phytosterins are for the most part cyclic alcohols of high molecular weight, which contain neither nitrogen nor phosphorus. The constitution of phytosterins has not yet been made clear.¹ The sitosterin of wheat and rye seedlings probably has the composition $C_{27}H_{45}OH$, and the phytosterin of beets is an alcohol with the empirical formula $C_{28}H_{45}OH$. There is only one double bond in cholestrin.² Although the phytosterins are entirely unlike the phosphatides in chemical respects, they seem to resemble them physiologically. Both groups of substances are included in the term "lipoids." The amount of phytosterin increases considerably during the germination of seeds in darkness.³ On the contrary, the content of phytosterin is very low in green seedlings. Still the phytosterins, like the phosphatides, are very generally distributed in small quantities. They have also been found in bacteria and fungi.

Analytical Methods.—The best way to prepare phytosterins is to extract the plant material with ether and saponify the extract with alcoholic potassium hydroxide to destroy the fats and phosphatides. The phytosterins are again extracted with ether and place in boiling alcohol. When cool, they separate in crystalline form.⁴ There are various sharp color tests for phytosterins, among which the following will be mentioned.

1. A bluish-red color is produced by shaking a solution of phytosterin in chloroform with an equal volume of concentrated sulphuric acid.⁵
2. If a solution of phytosterin in acetic anhydride is added in drops to sulphuric acid, the result is a violet color which soon changes to a green color.⁶
3. An acetic acid solution yields a red color with an orange-yellow fluorescence after heating with acetyl chloride and zinc chloride.⁷

Wax is a mixture of various fats, acids, phytosterins, hydrocarbons and other substances can therefore be regarded only as a collective idea. The melting point of plant waxes is higher than that of the other lipoids. The physiological rôles of the waxes also has nothing to do with those of the phosphatides and phytosterins. Wax is usually formed on the surface of cutaneous tissue and the formation of wax may be similar to the formation of cork and cuticular substance. As a rule, the chemical transformations of incrusting substances have been studied only very slightly.

5. ORGANIC CARBOXYLIC ACIDS

In fats and phosphatides, most of the carboxylic acids are esterified with alcohols. Still various other acids are common in plants, either in the free

¹ The latest information concerning the constitution of sterins can be found in **Windaus**. Ber. chem. Ges. 52: 162. 1918; **Idem**. Nachr. v. Kgl. Ges. d. Wiss., Göttingen, Math.-physik. Kl., p. 237. 1919. **Idem und Rahlen**. Zeitschr. f. physiol. Chem. 101: 223. 1918.

² **Tschugajew, L. und Kock**. Liebigs Ann. d. Chem. 385: 352. 1911.

³ **Schulze, E. und Barbieri**. Jour. f. prakt. Chem. 25: 159. 1882.

⁴ **Schulze, E. und Barbieri**. loc. cit.; **Ritter, E.** Zeitschr. f. physiol. Chem. 34: 430. 1902.

⁵ **Salkowski, E.** Pflügers Arch. f. ges. Physiol. 6: 207. 1872; **Hesse, O.** Liebigs Ann. d. Chem. 211: 273. 1878.

⁶ **Liebermann, C.** Ber. chem. Ges. 18: 1803. 1885.

⁷ **Tschugajew, L.** Zeitschr. f. angew. Chem., p. 617. 1900.

condition or in the form of salts. They are not indispensable constituents of the living protoplasm but they often play an important physiological rôle.

In biochemical respects, formic, acetic and butyric acids are noteworthy volatile acids.

Formic acid HCOOH . A thin liquid with a pungent odor; m.p. $+9^\circ$; boiling point 101° . Characterized by its aldehydic properties: it reduces silver salts and gives a red color with Schiff's reagent. Decomposed by oxidation. The lead salt is only slightly soluble in water. Some investigators consider that formic acid is an intermediate product of photosynthesis which serves as a transition stage from carbonic acid to formaldehyde (*cf.* Chap. II).

Acetic acid $\text{CH}_3\text{—COOH}$. M.p. 16.5° , boiling point 118° . Very hard to oxidize. The silver salt is somewhat soluble in water. Acetic acid is found in various fruits and plant juices but it is especially important as the principal product of acetic acid fermentation (Chap. VIII).

Butyric acid $\text{CH}_3\text{—CH}_2\text{—CH}_2\text{—COOH}$. Boiling point 162° . Is also found in some plants but is especially noteworthy as the principal product of butyric acid fermentation (Chap. VIII). Changes to acetic acid by strong oxidation. The yellow silver salt is only slightly soluble in water.

Glycolic acid $\text{CH}_2\text{OH—COOH}$. Occurs in various plants that are rich in sugar; m.p. $78\text{--}80^\circ$. Forms a slightly soluble copper salt.

Glyoxylic acid CHO—COOH has been discovered in different plants. Has aldehydic properties. Phenyl hydrazone decomposes at 137° . The free acid is sirupy but it yields a very characteristic basic calcium salt $(\text{C}_4\text{H}_5\text{O}_8)_2\text{Ca}_3$.¹

dl-Lactic acid $\text{CH}_3\text{—CHOH—COOH}$. Syrup. Boiling point, 119° at 14 mm. Soluble in water, alcohol and ether. Its almost insoluble zinc salt $(\text{CH}_3\text{—CHOH—COO})_2\text{Zn} + 3\text{H}_2\text{O}$ is very characteristic. The three molecules of water of crystallization distinguishes the zinc salt of dl-lactic acid of fermentation from that of d-lactic acid which crystallizes out with two molecules of water. Lactic acid has been found in many plants. Blackberry leaves² contain rather large amounts of it. It is especially known as the principal product of lactic acid fermentation.

Pyroracemic acid³ $\text{CH}_3\text{—CO—COOH}$. Syrup. Boils at $60\text{--}65^\circ$ at 14 mm. Soluble in ether. The zinc salt has the same properties as that of lactic acid and likewise has 3 molecules of water of crystallization. Pyruracemic acid differs sharply from lactic acid by its ketone properties. It reduces silver salts, unites with sulphites³ and yields a phenyl hydrazone (m.p. 192°) with phenyl hydrazine. Characterized by its barely soluble pyruracemic-acid-para-nitro-phenyl hydrazone, $\text{NO}_2\text{—C}_6\text{H}_4\text{—NH—N=C(CH}_3)_3\text{—COOH}$, with a dark yellow color and m.p. $219\text{--}220^\circ$.⁴

¹ Böttiger. Liebig's Ann. d. Chem. 198: 206. 1879.

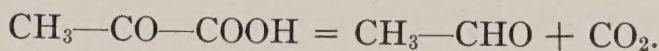
² Franzen, H. und Keyssner. Zeitschr. f. physiol. Chem. 115: 270. 1921; 116: 166. 1921; Franzen, H. und Stern. Ibid. 121: 195. 1922.

³ This acid is also known as pyruvic acid.—Ed.

³ Clewing. Jour. f. prakt. Chem. (2) 17: 241. 1878; Böttiger. Ber. chem. Ges. 15: 892. 1882.

⁴ Bamberger. Ber. chem. Ges. 32: 1806. 1899; Hyde. Ibid. 32: 1813. 1899.

Pyroracemic acid is an intermediate product of alcoholic fermentation and of other physiological conversions of sugar. Yeast splits pyroracemic acid into acetaldehyde and carbon dioxide without the addition of water (p. 53).



The unsaturated monobasic carboxylic acids are chiefly cleavage products of fats and alkaloids. Only one of them will be mentioned here and that is

Sorbic acid $\text{CH—CH=CH—CH=CH—COOH}$; m.p. 134° . In berries of the mountain ash.

The monobasic carboxylic acids just described are apparently prominent as products of intermediate metabolism, but they do not accumulate in the seed-plants in large quantities. On the contrary, the dibasic acids are often found in large amounts in seed-plants. Under certain conditions, some molds are also able to produce very considerable amounts of acid. The dibasic acids common in plants are chiefly solid, crystalline substances, very soluble in water and most of them in alcohol and ether as well. They yield only slightly soluble lead and calcium salts.

Oxalic acid $\text{COOH—COOH} + 2\text{H}_2\text{O}$ has the m.p. 98° but the anhydrous acid melts at 189° . Easily oxidized to CO_2 and water when held in aqueous solution. Characterized by its calcium salt which is practically insoluble not only in water but also in acetic acid. It dissolves only in mineral acids. Oxalic acid is very common in the plant kingdom, both free and in the form of its salts (oxalates). Crystals of calcium oxalate are found in many plants and are often deposited in specially adapted cells. These crystals often have the form of druses or of raphides. The latter are bundles of long, needle-like crystals. Single large crystals as well as crystal sand are often seen. The bark of some trees¹ and many succulents are very rich in calcium oxalate. As high as 65% of calcium oxalate is found in lichens.² This salt is also found in algae and in the higher fungi.³ Crystals of it are often seen in cell walls. The formation of oxalic acid by molds will be considered later.

Succinic acid $\text{COOH—CH}_2\text{—CH}_2\text{—COOH}$. Melts at 185° . Found in small quantities in many plants, especially in currants.⁴ Considerable amounts of it are formed in alcoholic fermentation by yeast as the result of deamination of glutamic acid (p. 309).

Suberic acid $\text{COOH—(CH}_2)_6\text{—COOH}$ has been isolated from cork.

l-Malic acid $\text{COOH—CH}_2\text{—CHOH—COOH}$ melts at 100° ; $[\alpha]_D = -5.17^\circ$. Decomposes easily in aqueous solution, especially in the light. The silver salt decomposes when warmed and deposits metallic silver. Malic acid also reduces palladium chloride. d-Malic acid and dl-malic acid, which do not occur in nature, are likewise known. Strange to say, the malic acid formed in succu-

¹ Heindrick, J. The Analyst. 32: 14. 1907; cf. also Smith. Proc. Roy. Soc. N. S. Wales. 1905.

² Braconnot. Ann. de chim. et de physique (2) 28: 318. 1825; cf. also Errera. Bull. Acad. Roy. Belgique (3) Vol. 26. 1893.

³ Bambeke, Ch. v. Bull. Acad. Roy. Belgique, p. 167. 1914; Patouillard, N. Rev. Mycol. 4: 87, 208 and 213. 1882; 5: 167. 1886; Woronin. Bot. Zeit. 38: 425. 1880; Leitger. Sitzungsber. d. Akad. Wien, Math.-natur. Kl. (I) 96: 13. 1888; and others.

⁴ Franzen, H. und Helwert. Zeitschr. f. physiol. Chem. 124: 65. 1922.

lents does not seem to be identical with the stereo-isomeric forms. However, the latest studies have shown that this "*Crassulaceae* malic acid" does not exist; it was found to be impure ordinary malic acid.¹ Malic acid becomes quantitatively prominent especially in northern latitudes. Thus only malic acid was found in berries of the mountain ash.² It also predominates in mountain cranberries, bilberries,³ cherries,⁴ plums,⁵ tomatoes⁶ and other fruits. Large quantities of malic acid are also contained in the vegetative organs of various plants, especially the succulents. In *Agave* the content of malic acid is 8% of the dry weight.⁷ In *Sedum azureum* the following amounts of oxalic acid in percent of dry substance have been measured:⁸

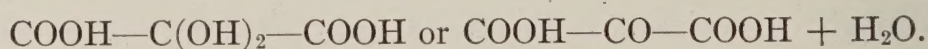
Date	Oxalic Acid	Malic Acid
May 25.....	1.82%	7.62%
June 17.....	0.48%	8.73%
June 27.....	2.07%	8.42%
July 8.....	0.74%	10.13%
July 29.....	0.35%	7.72%

The other dibasic acids are present in succulents in very small amounts. All the acid-forming plants of our latitudes contain malic acid.

d-Tartaric acid $\text{COOH}-\text{CHOH}-\text{CHOH}-\text{COOH}$. Melts at $168-170^{\circ}$; $[\alpha]_D = +15.06^{\circ}$. Characterized by the barely soluble acid potassium salt and the neutral calcium salt which, unlike calcium oxalate, is very soluble in acetic acid.

Tartaric acid is common in plants of southern lands. For example, it occurs in grapes, apricots and other succulent fruits. Like malic acid, it has also been found in small amounts in the higher fungi.⁹ In addition, grapes contain the dl-tartaric acid, or the so-called racemic acid, with the m.p. $205-206^{\circ}$.

Mesoxalic acid



Melts at 115° . Has ketone properties. Reduces silver salts. With phenyl hydrazine it yields a phenyl hydrazone which melts and decomposes at $165-167^{\circ}$. Mesoxalic acid, along with glycolic and glyoxylic acid, has been isolated from *Medicago sativa* and is probably important as an intermediate product of respiration.¹⁰

¹ Franzen, H. und Ostertag. Ber. chem. Ges. 55: 2995. 1922; Idem. Zeitschr. f. physiol. Chem. 122: 263. 1922.

² Idem. Zeitschr. f. physiol. Chem. 119: 150. 1922; however, cf. also Lippmann, E. O. Ber. chem. Ges. 55: 3038. 1922.

³ Rochleder, F. Ber. chem. Ges. 3: 238. 1870; Franzen, H. und Helwert. Zeitschr. f. physiol. Chem. 122: 46. 1922.

⁴ Feder, E. Pharmakol. Centralbl. 53: 1321. 1912.

⁵ Mercadante, M. Ber. chem. Ges. 8: 822. 1875.

⁶ Bot. Militär-med. Jour., Vol. 154. 1885 (Russian).

⁷ Zellner, J. Zeitschr. f. physiol. Chem. 104: 2. 1918.

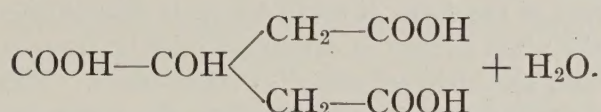
⁸ André, G. Compt. rend. 140: 1708. 1905; cf. also Branhofer und Zellner. Zeitschr. f. physiol. Chem. 109: 12. 1920.

⁹ Ordonneau. Bull. Soc. Chim. (3) 6: 261. 1891; Heckel et Schlagdenhaufen. Jour. de phar. et de chim. 19: 11. 1889; Husemann-Hilger. Pflanzenstoffe. 2nd Ed., p. 262; Franzen, H. und Kaiser. Zeitschr. f. physiol. Chem. 129: 80. 1923.

¹⁰ Euler, H. v. und Bolin. Zeitschr. f. physiol. Chem. 61: 1. 1909.

Fumaric acid $\text{COOH}-\text{CH}=\text{CH}-\text{COOH}$. Melts at 286° in fused capillaries. The silver salt dissolves a little in cold water. Fumaric acid has been found in various *Fumariaceae* and *Papaveraceae*, lichens and fungi. The mold *Aspergillus fumaricus* changes sugar into different acids of which 60–70% is fumaric acid.¹ Fumaric acid can be formed from acetic acid by way of the intermediate stage of succinic acid.² The stereo-isomeric maleic acid has not been found in plants and cannot be used as food by microorganisms.

Citric acid



Melts at 153° . The calcium salt is more soluble in cold water than in hot water.

Citric acid is very common in plants, especially in southern regions. In lemons the content of citric acid goes as high as 9% of the dry weight.³ Citric acid is also contained in the juice of mulberries⁴ and in a few other succulent fruits, and likewise in many higher fungi. The "citric acid fermentation" of molds will be considered later.

Analytical Methods.^h For qualitative demonstrations it is advisable⁵ to prepare the following fractions of acids: 1. Volatile acids. 2. Acids which are precipitated from neutral solution by lead acetate. 3. Ether-soluble acids which are not precipitated by lead acetate.

The extract of plant material is neutralized with NaOH and concentrated on the water bath (it is best to lower the pressure). Some sulphuric or phosphoric acid is then added and the whole is distilled by steam distillation. In the presence of lactic or pyroracemic acid, traces of these pass into the distillate with the volatile acids. The neutralized and perhaps concentrated distillate is tested for formic acid (aldehyde reactions and the formation of the insoluble lead salt). The formic acid is then decomposed by boiling with mercuric oxide and distilling with steam from acid solution. The second distillate is made neutral, concentrated as much as possible and precipitated with silver nitrate by degrees. Fractioning is facilitated by the fact that silver acetate is pure white but silver butyrate is yellowish. Another method is to precipitate the acetic acid, in the presence of ethyl alcohol, in the form of the sodium salt and filter. In the filtrate the butyric acid is precipitated as the silver salt.

Quantitative determinations of the volatile acids require the use of two aliquot portions of the distillate. In one portion the formic acid is determined by the methods described in Chap. II. In the other portion the formic acid is destroyed by the method just described and the amounts of acetic and butyric acid are measured as follows. The solution is titrated accurately with 0.1 N barium hydroxide, the solution evaporated and the percentage of barium determined in the salt mixture. From this the amounts of acetic and butyric acid are calculated by Windisch's method.⁶

Oxalic, tartaric, citric and malic acids are precipitated by lead acetate. The lead precipitate is decomposed with hydrogen sulphide and the filtrate from the lead sulphide is concentrated on the water bath (in the presence of formic acid it is better first to distill the filtrate with steam to remove the formic acid). Qualitative tests are then carried out for the indi-

¹ Wehmer, C. Ber. chem. Ges. 51: 1663. 1918; Ehrlich, F. Ibid. 44: 3737. 1911; 52: 63. 1919; Butkewitsch, W. und Fedoroff. Biochem. Zeitschr. 206: 440. 1929.

² Butkewitsch und Fedoroff. Biochem. Zeitschr. 207: 302. 1929.

³ Lüthrig, H. Zeitschr. f. Untersuch. d. Nahrungs- und Genussmittel. 11: 441. 1906; Zoller. Jour. Ind. Eng. Chem. 10: 364. 1918.

⁴ Wright und Patterson. Ber. chem. Ges. 11: 152. 1878.

^h This section on methods has been brought forward from its original position in the German edition where it was placed immediately after section 6 which now follows it.—Ed.

⁵ Kostytschew, S. An unpublished paper.

⁶ Windisch, K. Zeitschr. f. Untersuch. v. Nahrungs- und Genuss-mitteln. 8: 470. 1904.

vidual acids in order to determine which of them are absent. The test for oxalic acid consists in adding clear lime water or, after neutralization, a solution of calcium chloride. Calcium oxalate is insoluble in acetic acid. Tartaric acid gives a lilac-red color with a solution of 1 g. of resorcin in 100 g. of conc. H_2SO_4 at 125° . Citric acid is oxidized to acetone-dicarboxylic acid $\text{COOH}-\text{CH}_2-\text{CO}-\text{CH}_2-\text{COOH}$ by permanganate. This compound gives a characteristic precipitate with mercuric sulphate.¹ Malic acid reduces palladium chloride.

It is often very difficult to separate the non-volatile acids for quantitative determinations of the individual substances or to prepare the characteristic derivatives, especially with a mixture of several acids. The following method of Franzen² is undoubtedly the best one. The acids are fractionated in the form of ethyl esters or dihydrazides. Schmalfuss³ advises that all the acids from the plant extract or expressed juice be transferred to ether in the presence of 20% sulphuric acid and then separated as ethyl esters. This worker has worked out excellent micro tests for plant acids, described fully in the paper cited. In investigations of molds, tartaric and malic acid need not be considered but it is very important to separate the citric acid and the oxalic acid. This can be done fairly well by the two methods given by Butkewitsch.⁴ The calcium salts of both acids are dissolved in dilute HCl and the oxalic acid is then precipitated as the calcium salt with sodium acetate. The other method is to precipitate both acids as calcium salts and then to dissolve the calcium citrate in 2-2.5% HCl. Calcium oxalate is said to be dissolved very little in such dilute hydrochloric acid. On the other hand, it is not possible to dissolve all the calcium citrate with acetic acid in the presence of calcium oxalate.

As for the malic acid, it is rather easy to separate it from other acids because calcium malate is relatively soluble in water. After the removal of the other acids, the malic acid is determined quantitatively by reduction with palladium chloride:⁵ 0.294 g. palladium corresponds to 1 g. of malic acid.

To determine the succinic acid, the hydroxy acids are first decomposed by boiling with permanganate in sulphuric acid solution. The succinic acid is then extracted with ether, transferred to aqueous solution, neutralized carefully, precipitated with 0.1 N solution of silver nitrate and the excess silver titrated. 1 cc. of the silver solution corresponds to 0.0059 g. of succinic acid.

If the mixture of acids is precipitated with lead acetate, the soluble lead salts of lactic and pyroracemic acid remain in solution. Hence, it is advisable to isolate these acids as esters. It is best to change the lactic acid to the zinc salt. If the solution is highly colored, it is boiled with lead hydroxide and filtered through a hot-water funnel. The lead in the filtrate is removed with H_2S and the lead sulphide filtered off. The new filtrate is concentrated and treated with zinc carbonate, whereupon the barely soluble zinc lactate soon forms a precipitate. For the quantitative determination,⁶ the lactic acid is decomposed with permanganate in sulphuric acid solution and the acetaldehyde which forms at the same time is distilled off. In the distillate the acetaldehyde is fixed with bisulphite and titrated with iodine.⁷ The pyroracemic acid is similar to lactic acid in its properties but it differs sharply by the presence of the carbonyl group. It reduces silver salts and even in the cold yields the characteristic para-nitro-phenyl hydrazone. The pyroracemic acid can also be determined quantitatively in this form. The Zn salt and the Ca salt of the half carbazone of pyroracemic acid are especially characteristic.⁸

¹ Denigès, G. *Ann. de chim. et de physique* (VII) **18**: 382. 1899. [The exact method of preparing this "Denigès reagent" is to dissolve 1 gram of mercuric oxide in a mixture of 4 cc. conc. H_2SO_4 and 20 cc. distilled water.]

² Franzen, H. und Helwert. *Zeitschr. f. physiol. Chem.* **122**: 46. 1922.

³ Schmalfuss, H. und K. Keitel. *Zeitschr. f. physiol. Chem.* **138**: 156. 1924.

⁴ Butkewitsch, W. *Biochem. Zeitschr.* **131**: 327 and 338. 1922. Cf. also Sommer, H. M. and E. B. Hart. *Jour. Biol. Chem.* **35**: 313. 1918.

⁵ Hilger, A. und H. Ley. *Zeitschr. f. Untersuch. v. Nahrungs- und Genussmitteln.* **2**: 795. 1899; Hilger, A. *Ibid.* **4**: 49. 1901.

⁶ Fürth, O. v. und D. Charnass. *Biochem. Zeitschr.* **26**: 207. 1910.

⁷ Ripper. *Monatsh. f. Chem.* **21**: 1079. 1900.

⁸ Kostytschew, S. und S. Soldatenkow. *Zeitschr. f. physiol. Chem.* **176**: 287. 1929.

6. THE TRANSFORMATIONS AND PHYSIOLOGICAL IMPORTANCE OF THE CARBOXYLIC ACIDS

The percentage of organic acids in seed-plants fluctuates widely. As a rule, there is more acid in young leaves than in older leaves.¹ The amount of malic acid in the succulents is not constant. It accumulates at night but decreases throughout the day.² This depends on the oxidation of malic acid to carbonic acid in the light, and the use of the latter acid during photosynthesis.

It is therefore evident that malic acid in plant cells can be oxidized to CO₂. It is still easier to oxidize the oxalic acid there. An enzyme for this purpose has been found in plants³ and it proves to be very widely distributed.⁴

Although the plant acids can undoubtedly be burned to CO₂ and H₂O under suitable conditions, it would be premature to conclude from this that these processes of oxidation are a part of normal plant respiration, as different investigators have assumed, but before the nature of the oxidation processes in plant cells was understood and the connection between alcoholic fermentation and oxygen respiration established by experimental investigations. From the present standpoint, such theories are neither theoretically nor experimentally justified. Years ago they found much favor and were admitted into modern texts. Thus there is a consideration of the physiological conversions of plant acids even in the recent edition of the basic hand-book "Biochemie der Pflanzen" by F. Czapek,⁵ in the section on plant respiration. However, it will be evident from what follows that this conception is hardly free from objections.

A. Mayer⁶ long ago expressed his opinion that in plant respiration under certain conditions, malic and oxalic acid might be formed in place of carbon dioxide. That Mayer's views depend on obsolete ideas, is evident from the fact that he also assumed the reverse process, *i.e.*, a *direct* synthesis of sugar from plant acids by light, whereby carbon dioxide is not even temporarily formed. Mayer's theory was then perfected and extended by Warburg⁷ and by Puriewitsch.⁸ These workers assumed that sugar is gradually oxidized to form plant acids containing more oxygen. Finally oxalic acid is said to be formed as the preliminary to complete combustion. At that time oxalic acid was erroneously taken to be the plant acid which accumulates in excess. In reality, malic acid is the most important product of the metabolism of fleshy fruits and succulents, as explained above.

Since deaminization has been recognized as a widespread process we know that the cleavage products of proteins may be regarded as parent substances of various nitrogen-free plant materials. The author of this book believes that

¹ Kraus, G. Abh. d. Naturforscherges. Halle, Bd. 16. 1884; Astruc, A. Compt. rend. 133: 491. 1902; Charabot et Hébert. *Ibid.* 136: 1009. 1903.

² Kraus, G. *loc. cit.*; Lange, P. Diss. Halle. 1886; Puriewitsch, K. The synthesis and decomposition of organic acids in seed-plants, 1893 (Russian); and others.

³ Zaleski, W. und Reinhard. Biochem. Zeitschr. 33: 449. 1911; Palladin, W. und Lowtschinowsky. Mitt. russ. Akad. d. Wiss., p. 937. 1916 (Russian).

⁴ Stahelin, M. Biochem. Zeitschr. 96: 1. 1919.

⁵ Czapek, F. Biochemie der Pflanzen. 2nd. ed. 3: 65-110. 1921.

⁶ Mayer, A. Landwirt. Versuchs-Stationen. 34: 127. 1887.

⁷ Warburg, O. Untersuch. d. bot. Inst. Tübingen. 2: 53. 1886.

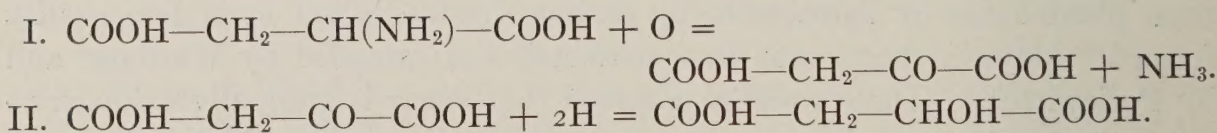
⁸ Puriewitsch, K. *loc. cit.*

he can assume that the common plant acids represent either transformation products of amino acids, or either normal or by-products of the incomplete change of sugar to amino acids during the synthesis of proteins.¹

Such a strongly oxidized substance as oxalic acid can of course be derived from protein as well as from sugar. It is known, for one thing, that a considerable amount of oxalic acid is formed in the treatment of proteins with nitric acid. Wehmer² showed that some molds produce enormous quantities of oxalic acid at the expense of sugar, yet this investigator refers expressly to the fact that part of the oxalic acid is formed not from sugar but from protein substances. It has also been found that oxalic acid is formed in fungus cultures with peptone as the sole nutrient.³ From single amino acids as the sources of carbon the same product was observed. Benecke⁴ has proved that oxalic acid is often formed in the synthesis of proteins from nitrates. According to some unpublished experiments, *Aspergillus niger* also produces oxalic acid in the synthesis of protein from sugar and nitrates. The copious formation of oxalic acid during the regeneration of protein from asparagine in seed-plants is only the result of an oxidation of the carbon framework of asparagine, according to Petrow.⁵

If it is conceivable that oxalic acid may be formed from protein as well as from sugar, the possibility of the formation of other organic acids from the cleavage products of the proteins appears to be still greater. Yet the formation of succinic acid from sugar has not been recorded, though its formation from glutamic acid is established beyond question.⁶

No nitrogen-free acids are formed when yeast works on aspartic acid; this was explained on p. 303. S. Kostychev and L. Frey,⁷ were able to obtain the formation of malic acid by yeast, which is to be explained as follows:



These reactions appear to take place in the case of some molds and they may also be possible in seed-plants. In this way we are justified in assuming that both oxalic and malic acid may come from aspartic acid or asparagine. Still most of the malic acid probably comes from sugar, but this represents not an intermediate phase of the normal respiratory process but a stage in the formation of asparagine. Actually an increased formation of malic acid or malic acid monoamide was demonstrated by Schulow and Petrow⁸ for germinating

¹ On this point see especially Ruhland, W. und K. Wetzel. *Planta*. 1: 558. 1926; 7: 503. 1929.

² Wehmer, C. *Bot. Zeit.* 49: 233. 1891; *Ber. bot. Ges.* 24: 381. 1906; *Centralbl. f. Bakt.* (II) 3: 102. 1907.

³ Butkewitsch, W. *Biochem. Zeitschr.* 129: 445. 1922.

⁴ Benecke. *Bot. Zeit.* 61: 79. 1903.

⁵ Petrow, G. G. The assimilation of nitrogen by seed-plants in the light and in darkness. 1917 (Russian).

⁶ Ehrlich, F. *Biochem. Zeitschr.* 18: 391. 1909.

⁷ Kostytschew, S. und L. Frey. *Zeitschr. f. physiol. Chem.* 1925.

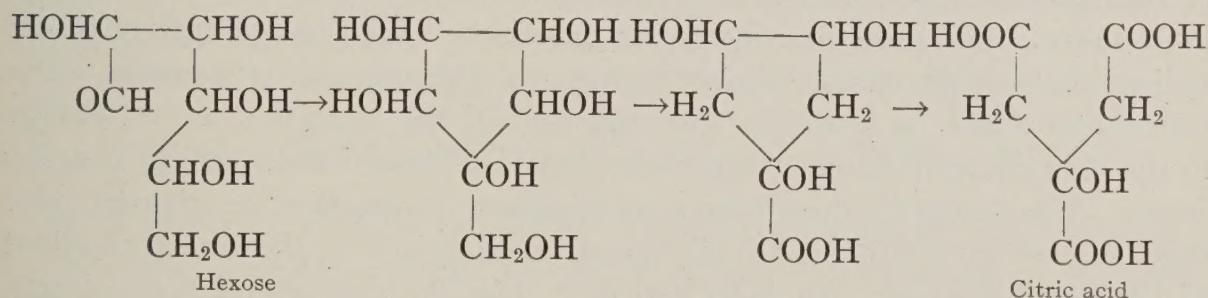
⁸ Schulow, J. Investigations in the field of the physiology of nutrition of the higher plants. 1913 (Russian); Petrow, G. G. *loc. cit.*

seed-plants. Prianischnikow and his students¹ also observed a considerable increase in the amount of asparagine in seedlings after feeding salts of malic acid. Still more interesting in this connection are the studies in animal physiology by Embden and his co-workers² who have effected a regular transformation of the ammonium salts of α -keto- or α -hydroxy-acids into amino acids of the corresponding constitution by experiments in which blood is passed through isolated liver. This work indicates the course of the biological synthesis of amino acids.

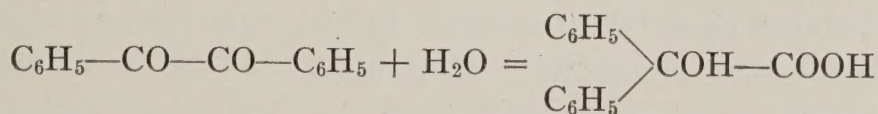
For these reasons it is most probable that most of the malic acid of seed-plants is formed in the process of formation or deaminization of asparagine. Frequently, perhaps because of a deficiency of nitrogen, all the malic acid cannot be worked into asparagine. The excess malic acid is then destroyed by oxidation processes leading to the formation of CO_2 , but this is not a direct or normal respiratory process. The oxidation of plant acids is much more like the so-called "protein respiration" which goes on in plants when sugar is lacking (*cf.* Chap. VIII).

In the case of the lower plants, only small amounts of malic acid are found at the most. It should also be noted that asparagine is never formed in the fungi and bacteria.

As for citric acid with its branched carbon chain, this acid cannot be formed from sugar by simple oxidation. There must be here either a synthesis from the cleavage products of sugar or an intramolecular rearrangement. For example, Butkewitsch³ believes that the following reactions underlie the formation of citric acid from sugar:



The author of this book considers another method of forming citric acid to be more probable. That is citric acid might be produced by the simultaneous oxidation and intramolecular rearrangement of sugar. In the same way, as in the case of the formation of benzylic acid from benzyl or glycolic acid from glyoxal where one phenyl or one hydrogen migrates to another carbon

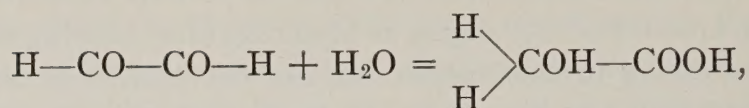


¹ Prianischnikow, D. *Tageb. d. 1 Kongr. russ. Bot.*, p. 65. 1921 (Russian); Smirnow, A. *Biochem. Zeitschr.* 137: 1. 1923.

² Embden, G. und E. Schmitz. *Biochem. Zeitschr.* 29: 423. 1910; 38: 393. 1912; Kondo, K. *Ibid.* 38: 407. 1912; Fellner. *Ibid.* 38: 414. 1912.

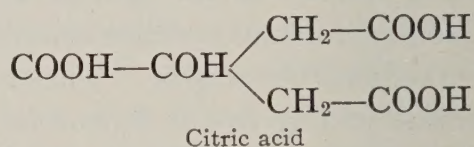
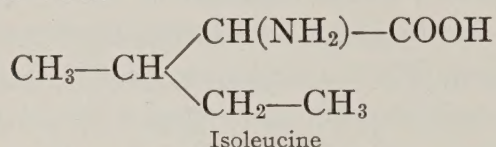
³ Butkewitsch, W. *Biochem. Zeitschr.* 145: 442. 1924.

or



so the carboxyl group of gluconic or saccharic acid can rearrange itself during the oxidation of glucose.¹

However, it might be possible to form citric acid by the synthesis of structural units of proteins through the intervention of oxidation. For example, it must be borne in mind that the carbon skeleton of isoleucine is identical with that of citric acid:



It is evident that citric acid might be formed from cleavage products of proteins after their deaminization. However, most of the citric acid comes from sugar, not by means of respiratory processes, but as a by-product of the formation of the structural units of proteins. It is certainly not very likely that sugar is oxidized in normal plant respiration with any such rearrangements as are required to form citric acid.

Thus we might draw the general conclusion that the plant acids which are formed in the largest quantities, such as malic, citric, etc., are not normal intermediate products of sugar respiration. If a complete oxidation of plant acids to carbon dioxide and water occurs under certain conditions, it seems to be a process which destroys substances which have lost their physiological importance. For example, this is the explanation for the combustion of the carbon framework of asparagine during the regeneration of protein. This conception might be tested by investigations of the formation of and change in the organic acids during the germination of seeds and the ripening of fleshy fruits. The changes in the nitrogenous substances during seed germination have been studied in detail but an analogous investigation of the transformations of the acids has only just been started.¹ For the ripening of fleshy fruits, however, only the formation and disappearance of the organic acids has been followed. The nitrogen exchange in fleshy fruits is still wholly unknown to us.

As for the variations in the content of acid in fleshy fruits, this process is very similar to that in succulents.² As a rule, when the fruit ripens the acid content decreases while the sugar content increases. However, there is no connection between these two processes. In some cases we find a simultaneous increase in both sugar and acids.³ Thus sugar is formed not from acids but

¹ Approximately the same hypothesis has been proved in part by work with *Aspergillus* and sugars. See Challenger, Subramaniam and Walker. Jour. Chem. Soc. (London), p. 200-208. 1927; Walker Subramaniam and Challenger. *Ibid.*, p. 3044-3054.—Ed.

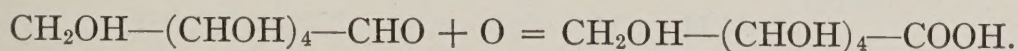
² Windisch und Dietrich. Wochenschr. f. Brauer. 35: 159. 1918; Lüers, H. Biochem. Zeitschr. 104: 30. 1920.

³ Warburg, O. Untersuch. d. bot. Inst. Tübingen. 2: 53. 1886.

³ Faminzyn, A. Ann. Oenol. Vol. 2. 1872.

from the reserve starch and the substances which are brought in. In regard to the carboxylic acids, they are often burned during the ripening process, to judge from the ratio of the gases in the respiratory gas exchange of ripening fruits.¹ Unfortunately we do not know the origin of most of the carboxylic acids in ripening fruits.

The organic carboxylic acids are also important in the metabolism of the molds. When fed on sugar, almost all molds produce a certain amount of carboxylic acids, especially oxalic and citric. The malic acid which predominates in seed-plants, is not produced by molds in appreciable amounts. If calcium carbonate is added to a sugar culture of the ordinary mold *Aspergillus niger* until the acids are neutralized, the fungus changes enormous quantities of sugar into oxalic acid;² still a certain amount of this acid always comes from the cleavage products of proteins. Moreover, it has been emphasized for a long time that some species of *Penicillium*, under certain growth conditions, produce large quantities of citric acid.³ Hence these fungi have been called species of *Citromyces*. However, it has been found recently⁴ that the genus *Citromyces* is a weak one because various kinds of molds produce citric acid. Butkewitsch⁵ has shown that citric acid in fungus cultures comes only from sugar and from this he concludes that citric acid is a normal product of the utilization of sugar by molds. However, Kostychev and Tschesnokow⁶ have proved that there is no connection between citric acid and the respiratory metabolism. Citric acid is formed only in periods of a rapid growth of the fungus and especially when proteins are being synthesized and then only when there is a sudden cessation of nitrogen assimilation which can be restricted either by the exhaustion of the nitrogenous substances in the substrate or by a complete saturation of the fungus with nitrogen. With a deficiency of mineral nutrients, *Aspergillus niger* produces much gluconic acid, the first product of the oxidation of sugar:⁷



It is hardly permissible to use the expressions "oxalic acid fermentation," "citric acid fermentation," etc. for the processes of forming acids. According to modern ideas, true fermentations are independent systems of coupled reactions which can be separated from living protoplasm and which, as sources of energy, involve the working up of enormous quantities of material. Our present knowledge of the production of carboxylic acids by molds can scarcely be harmonized with these conceptions.

¹ Gerber, C. Compt. rend. 124: 1160. 1897; Ann. Sci. Nat. Bot. (8) 4: 1. 1896.

² Wehmer, C. Bot. Zeit. 49: 233. 1891; Ber. bot. Ges. 24: 381. 1906; Centralbl. f. Bakt. (II) 3: 102. 1897; Benecke. Bot. Zeit. (II) 65: 73. 1907.

³ Wehmer, C. Ber. bot. Ges. 11: 333. 1893; Centralbl. f. Bakt. (II) 15: 427. 1906.

⁴ Currie, N. Jour. Biol. Chem. 31: 15. 1917; Molliard. Compt. rend. 168: 360. 1919; Elfving, F. Oefvers. af vetenskaps soc. förh. A. math. o. natur. 61: 1. 1920.

⁵ Butkewitsch. Biochem. Zeitschr. 131: 327 and 338. 1922; 136: 224. 1923; 142: 195. 1923; 145: 442. 1924; [see also citations in note 1 above and Bernhauer and Schön. Biochem. Zeitschr. 202: 164-179. 1928].

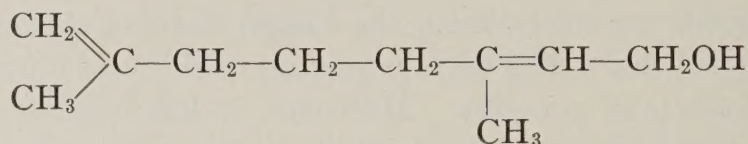
⁶ Kostytschew, S. und W. Tschesnokow. Planta. 4: 181. 1927.

⁷ Molliard. Compt. rend. 174: 881. 1922; 178: 41. 1924.

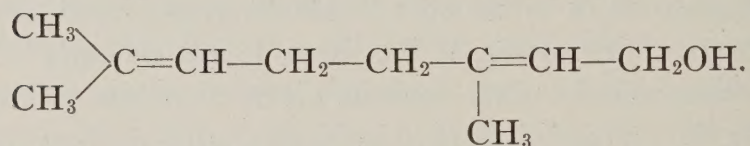
7. TERPENES, RESINS AND RUBBER

Terpenes are alicyclic methylated substances with a pleasant odor, which are volatile in steam and make up a large part of the so-called ethereal oils. They are contained in specific secretion reservoirs (druses).

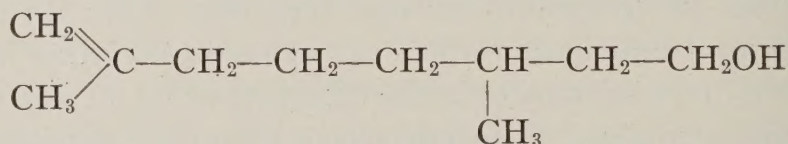
The terpenes form a large and chemically well defined group of substances. They are divided into types which readily change into each other. A few aliphatic substances are also numbered with the terpenes on account of their properties. These substances can be easily changed into cyclic terpenes.¹ The following are typical aliphatic terpenes.

Geraniol

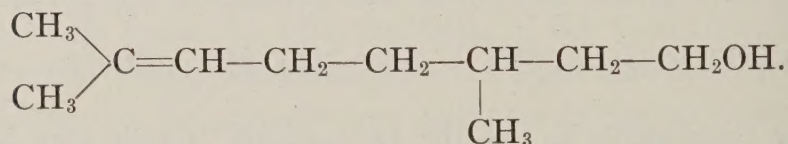
or



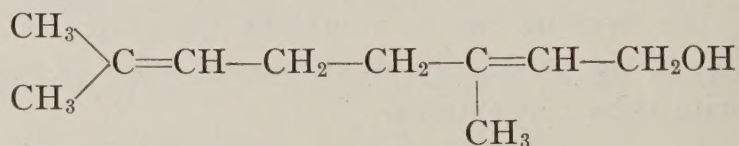
Boiling point 230°. Found in the ethereal oils of some species of *Andropogon* and in many other plants. Its acetic acid ester is the chief constituent of oil of *Eucalyptus* and also occurs in other plants.

Citronellol

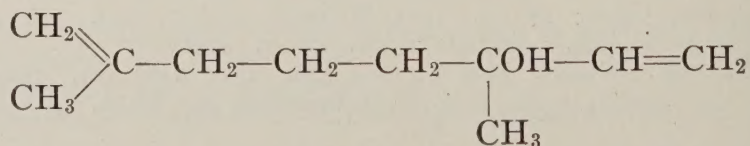
or



Boils at 225° and occurs in oils of roses and geranium and in other plant secretions.

Nerol

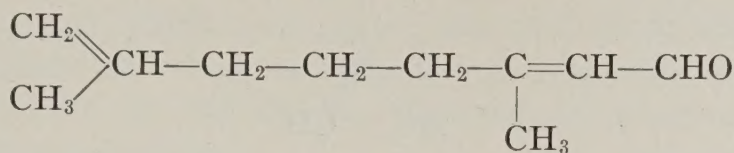
A stereo-isomeric form of geraniol. Boils at 225°. In oil of citrus, oil of roses, etc.

Linalool

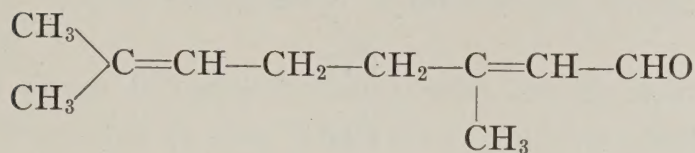
¹ Semmler, F. W. Ber. chem. Ges. 23: 1098, 2965 and 3556. 1890; 24: 201 and 682. 1891; and others.

Boils at 197–200°. In many ethereal oils.

Citral



or



Boils at 224° and is the aldehyde corresponding to the alcohol geraniol. Occurs in orange oil and in many other ethereal oils.

The cyclic terpenes are divided into various groups of which the following will be mentioned here:

1. Dipentenes.

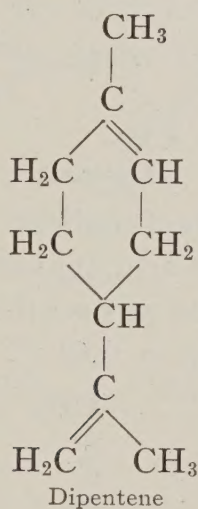
2. Pinenes.

3. Thujones.

The general formula for cyclic hydrocarbons of the terpene class is $\text{C}_{10}\text{H}_{16}$. These substances contain a six-membered or doubled ring plus side chains. The so-called hydroterpenes $\text{C}_{10}\text{H}_{18}$ and $\text{C}_{10}\text{H}_{20}$ are closely related to the terpenes. The grouping $\text{CH}_3-\text{C} \leq$ which often occurs in terpenes is replaced in some substances by the isomeric grouping $\text{CH}_2=\text{C} <$. Both isomeric forms occur together in natural products. The terpenes oxidize and become resins very easily.

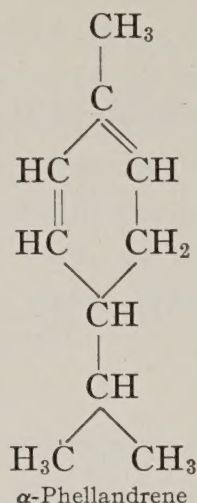
The following dipentenes will be mentioned:

Dipentene or dl-limonene

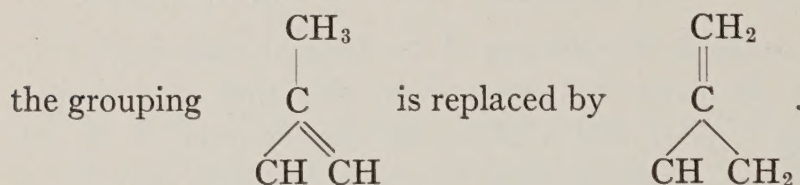
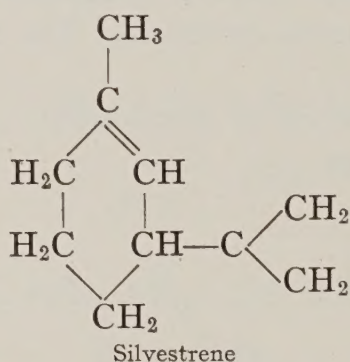


There are also the two optically active limonenes. Boils at 175–176°. $[\alpha]_D = \pm 123^\circ 40'$. The constitution of dipentenes, limonenes and other related substances has been made clear by the excellent syntheses by Perkin, Jr.¹ Both dipentene and the two limonenes can be prepared in large quantities from various ethereal oils.

¹ Perkin, Jr. Jour. Chem. Soc. (London) 85: 654. 1904; 93: 1416 and 1871. 1908; cf. also Deussen und Hahn. Ber. chem. Ges. 43: 519. 1910; Wagner, G. Ibid. 27: 1653 and 2270. 1894; Wallach. Liebigs Ann. d. Chem. 281: 127. 1894; and others.

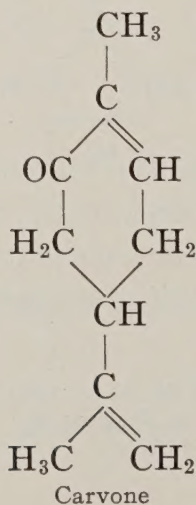
α -Phellandrene

Boils at 173–176°. Optically active. Found in many ethereal oils. A mixture of the two isomers is always found in natural products; one of them is represented by the accompanying formula while the other is the “pseudo form” in which

**Silvestrene**

Boils at 175–176°. In ethereal oils of conifers. An intense blue color is produced by dissolving silvestrene in acetic acid anhydride and adding a drop of concentrated sulphuric acid. Silvestrene was synthesized by Perkin.¹

Carvone. Boils at 230°. $[\alpha]_D = \pm 62.1^\circ$.



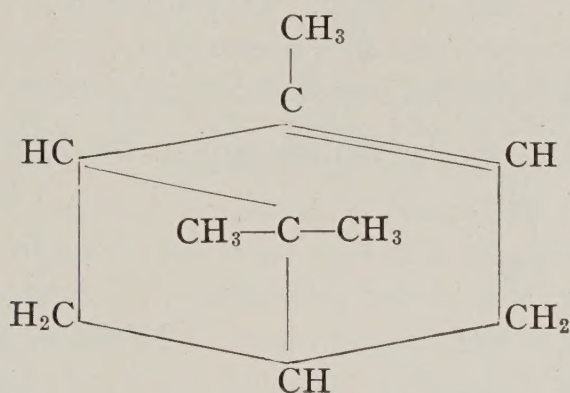
¹Perkin, Jr. and Tattersall. Jour. Chem. Soc. (London) 91: 480. 1907.

Carvone is a ketone of the dipentene group and occurs in its two optically active forms. Smells like caraway. Changed on heating to the isomeric carvacrol (see p. 350), which indicates the ease with which terpenes change to the benzene derivatives. Carvone is contained in the ethereal oils of *Carum* and other members of the *Umbelliferae*. In some plants carvone is linked with different molecular complexes.

Refer to the special manuals for the numerous other substances in the dipentene group.

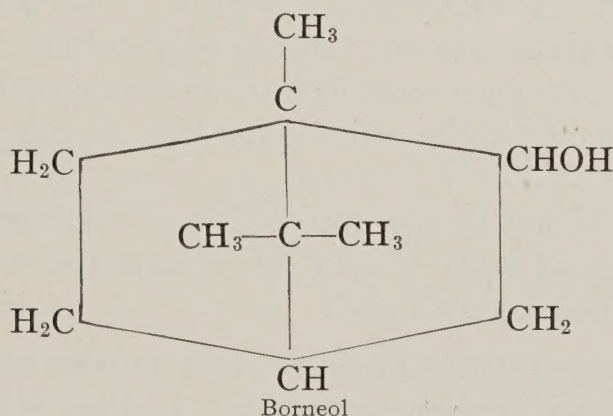
The pinene group includes terpenes with the so-called "bridge union" which divides the simple six-ring into a six-ring and a four-ring. Later it will be explained that the "bridge" can easily be dissolved and the pinene group is then changed to the dipentene group.

Pinene



is the principal constituent of ordinary oil of turpentine from various species of *Pinus*. Both its optically active forms are contained in the natural products. Boils at 156° . $[\alpha]_D = \pm 45^{\circ}$. The constitution of pinene is established with certainty.¹ The characteristic odor of oil of turpentine depends on an aldehyde-like oxidation product of pinene.²

Borneol

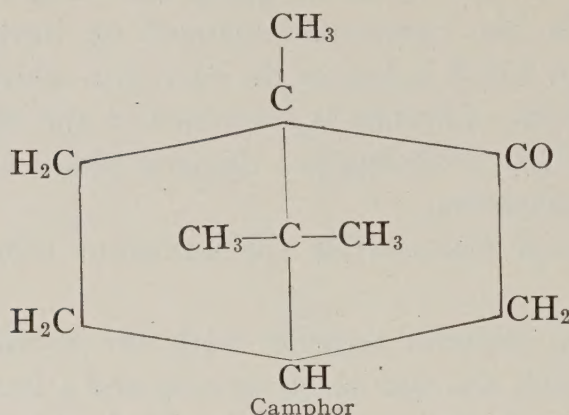


unlike pinene, has a straight "bridge" and therefore consists of two five-rings. Melts at 203° and boils at 212° . Occurs in many ethereal oils, partly in the free form and partly as esters. Very volatile.

¹ Wagner, G. Ber. chem. Ges. 27: 1636. 1894; Baeyer, A. v. Ibid. 29: 3. 1896.

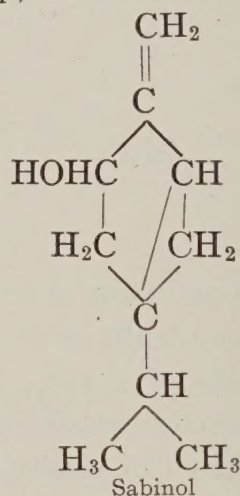
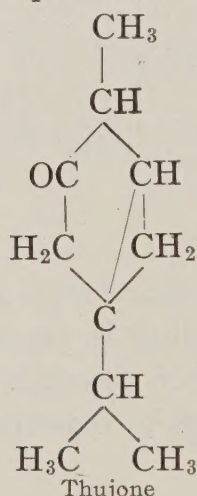
² Schiff, H. Chem. Zeit. 20: 361. 1896.

Camphor



is really the most important compound of the borneol group. Its constitution is known accurately.¹ Like borneol, camphor is a solid substance. Melts at 178° and boils at 204° . $[\alpha]_D = +45^{\circ}$. Is optically active only in the dissolved and liquid form. Camphor was once prepared only from the druses of *Laurus camphora*. Now it is artificially prepared from various terpenes (see below).

Among the terpenes of the thujone group, the following are of interest.



Thujone. Melts at 202° . $[\alpha]_D = +76.16^{\circ}$.² In ethereal oils of *Thuja*, *Salvia*, *Tanacetum*, *Matricaria*, etc. It can be seen from the formulae that the thujones differ from the dipentenes in that the double bond of the dipentenes is replaced in thujone by a bridge which divides the six-ring into a five-ring and a three-ring.

Sabinol is the alcohol of the "pseudo class" corresponding to the ketone thujone. It was found in the ethereal oils of *Cupressus* and *Juniperus sabina*.³ Dextrarotatory. Boils at 208° – 212° .

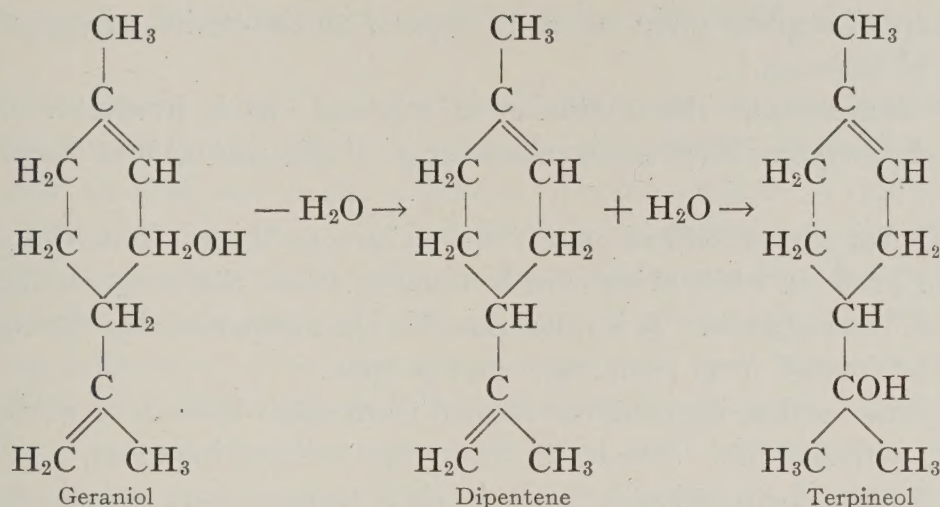
There are numerous other substances of the terpene type but it suffices here to name the foregoing typical compounds. It must be pointed out that all terpenes readily change from one form to another. It is especially important that aliphatic terpenes are easily changed to rings. This may be a general method of the physiological formation of cyclic substances, since cyclic terpenes easily change to benzene derivatives.

¹ Komppa. Ber. chem. Ges. 36: 4332. 1903; 41: 4470. 1908.

² Wallach. Liebigs Ann. d. Chem. 272: 99. 1892; 275: 159. 1893; 277: 159. 1893; 286: 90. 1895; 323: 333. 1902; and others.

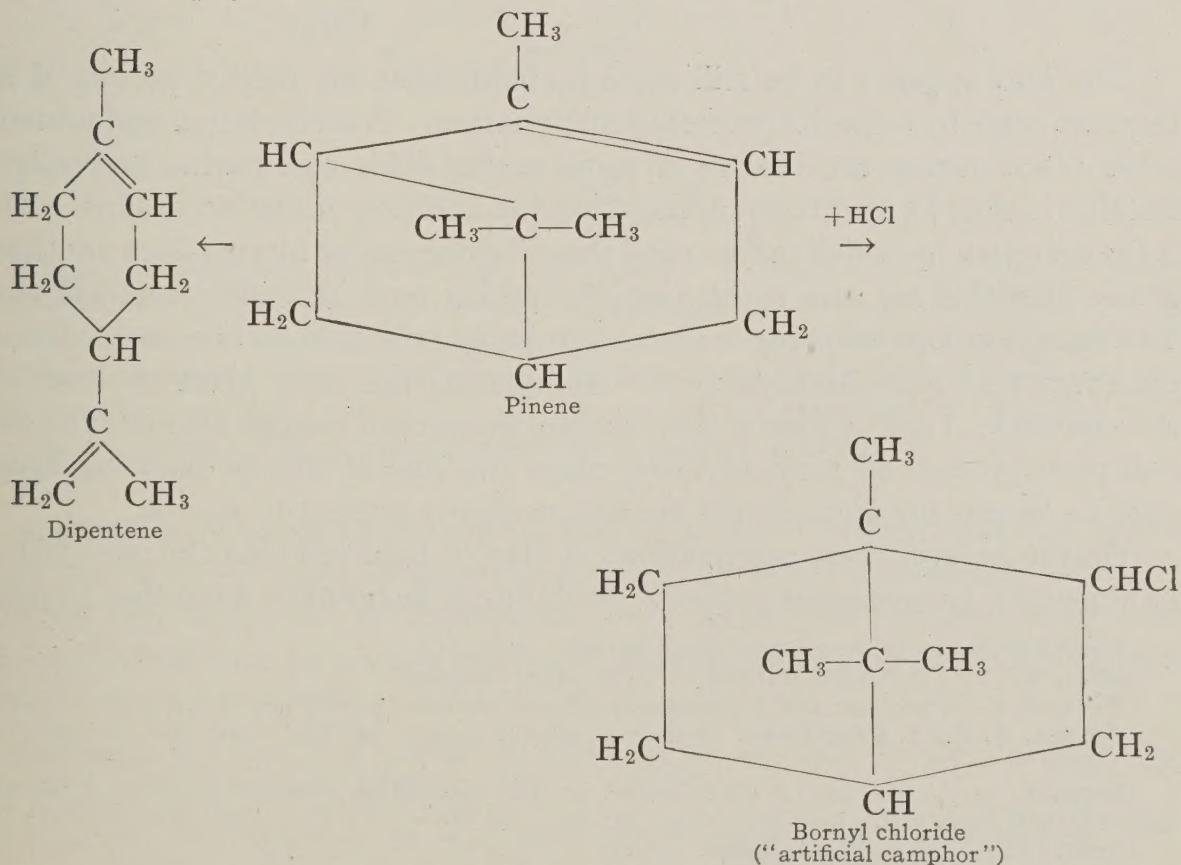
³ Fromm, E. Ber. chem. Ges. 31: 2025. 1898; 33: 1191. 1900; Semmler. Ibid. 33: 1459. 1900.

Both linalool and geraniol change to dipentenenes by the action of acid potassium sulphate or dilute acids:



On the other hand, pinene also undergoes a conversion to dipentene by boiling with dilute acids. Dry hydrochloric acid converts the pinene nucleus into the borneol nucleus by "straightening the bridge" (see formula below).

The formation of terpenes in plants is connected with important vital processes. The hypothesis is not precluded that the unstable forms of these compounds cannot be isolated because they are at once changed further in the physiological metabolism and thus never accumulate in appreciable amounts. According to this view, we know only the stable and hence the less vitally important substances in this class. The terpenes have been found in all groups of seed-plants and even in the liverworts. They do not seem to occur in plants without chlorophyll.

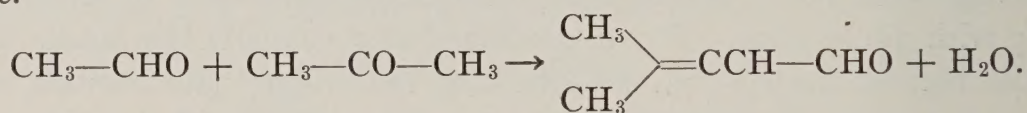


The physiological rôle of terpenes is unknown. We can only emphasize the ease with which they are oxidized. The distribution of ethereal oils in various parts of a given plant seems to depend on the action of oxygen in the formation of terpenes.¹

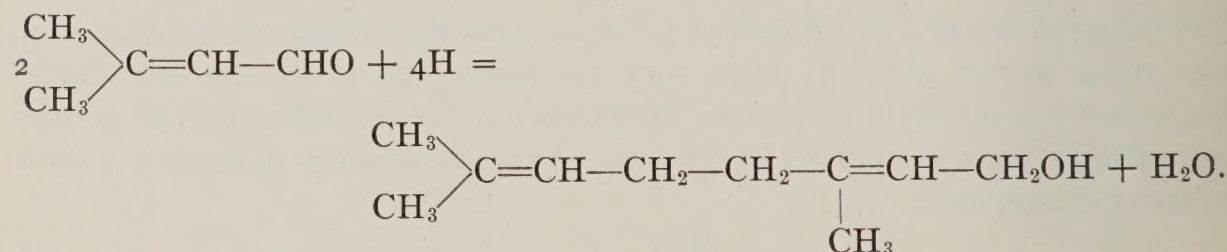
Nilov² showed that the liberation of ethereal oils is irregular. A single specimen of *Juniperus excelsa* can release 30 g. of ethereal oil into the air in one day.

Some investigators believe that terpenes are produced from the cleavage products of proteins while others are inclined to think that sugar is the parent substance of the terpenes. It is quite possible that terpenes, like the carboxylic acids, can be formed from both sugar and protein.

Euler³ believes that terpenes are formed from sugar by way of acetaldehyde which joins with acetone. The latter compound is found in plants.⁴ According to Euler, β -methyl-crotonaldehyde is then formed from acetaldehyde and acetone.



Geraniol is formed by condensation of two molecules of methylcrotonic aldehyde and reduction of the resultant product:



The idea appears to be still more probable that the methyl groups of the terpenes come by a special process of methylation. A methylation undoubtedly takes place during the change of some amino acids and purine derivatives. Betaine is thus formed from glycocoll by the addition of methyl groups. This is the only way by which caffeine and theobromine can be formed from xanthine. Many alkaloids are also synthesized by taking part in a direct methylation. The methyl groups of valine or leucine probably arise in the same way. Pictet⁵ considers that formaldehyde is the methylating factor in plant tissues. On the contrary, Trier⁶ believes that he can show that methyl alcohol, the first reduction product of formaldehyde, plays the rôle of the methylating agent. Specific factors for methylation are also probably present in plants.

On the basis of these explanations, it may be assumed that the methylation of terpenes is an analogous process. Still it must be borne in mind that terpenes

¹ Pigulewski, G. Jour. russ. Chem. Ges. 54: 262. 1923.

² Nilov, W. Arb. d. Nikitski Gartens in Krym. 1924 (Russian).

³ Euler, H. v. Grundlagen und Ergebnisse der Pflanzenchemie. 3: 219. 1904.

⁴ Palladin, W. und S. Kostytschew. Zeitschr. f. physiol. Chem. 48: 214, 1906; Ber. bot. Ges. 24: 273. 1906.

⁵ Pictet, A. Arch. sci. phys. et nat., Genève, p. 329. 1905; Idem. Arch. de pharmacie. 244: 389. 1906; Pictet, A. und Court. Ber. chem. Ges. 40: 377. 1907.

⁶ Trier. Über einfache Pflanzenbasen. 1912.

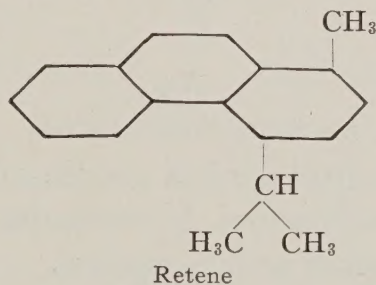
are formed in large quantities especially in plants containing chlorophyll. The plant produces formaldehyde, the methylating factor, in the light. Direct observations also show that light facilitates the formation of ethereal oils in plants.¹

The terms "terpenes" and "ethereal oils" are not synonymous, since various other substances, especially the so-called **resins** have been found in ethereal oils, in addition to the terpenes. These solutions of resins are often known as **balsams**. Resins are not single chemical compounds but mixtures of different kinds of components. Hence the classification of plant resins is not based on their chemical composition.² Resins are always amorphous and often are colored substances which are readily oxidized when exposed to oxygen. The so-called pathological flow of resin is especially striking. Just as in the case of the formation of gums mentioned in the preceding chapter, the flow of resin from the biochemical standpoint is a very interesting phenomenon which has unfortunately been investigated only very scantily.

Many resins are important in medicine and for technical uses. Different investigators have found uniform chemical compounds to be contained in resins. Various alcohols, tannins, acids and peculiar stable "resenes" have also been isolated from the resins. Among the resinols, *i.e.*, resin alcohols, we shall mention the **amyrines**³ which are similar to the terpenes and include α -amyrine (m.p. 180–181°) and β -amyrine (m.p. 193–194°). Substances much like the amyrynes have been isolated from the commercially important gutta-percha.

Resinotannols are probably condensation products of phenol derivatives and are yellow or brown in color. Some resinotannols yield picric acid when oxidized with nitric acid.

Resinol acids appear to be related to the terpenes. On distillation they usually give the hydrocarbon retene, a phenanthrene derivative:



Retene is an important constituent of coniferous tar. It melts at 98.5°, is slightly soluble in alcohol and very soluble in ether. It is characterized by tetra-brom-retene $C_{18}H_{14}Br_4$. We are justified in assuming that the resinol acids are retene derivatives. **Abietic acid** is an example of the resinol acids. It is the principal constituent of the American rosin and the resin of various conifers. Abietic acid contains one carboxyl group and the retene ring with its isopropyl group. The chemical constitution of **resene** is unknown.

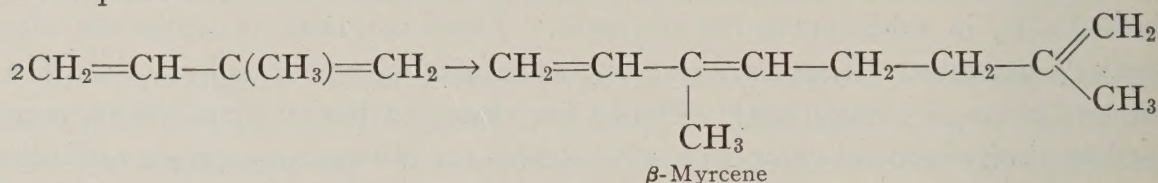
Rubber is contained in the latex of various plants. Industrial rubber comes from the latex of some tropical trees, such as *Hevea brasiliensis*, *Manihot Glaziovii*, *M. dichotoma*, *M. heptaphylla*, etc. A colloidal rubber is said to be prepared in the latex but undoubtedly this is a polymerization product of certain other constituents of latex.

¹ Lubimenko, W. Mitt. d. bot. Garten in Petersburg. Vol. 16. 1916 (Russian); Lubimenko and Fichtenholz. Mitt. d. wiss. Inst. v. Leshaft. Vol. 1. 1920 (Russian).

² Tschirch. Die Harze. 2nd. ed. 1906.

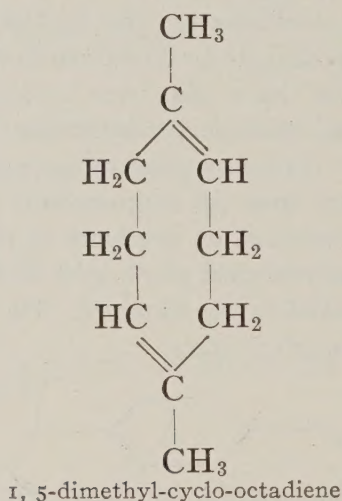
³ Vesterberg. Ber. chem. Ges. 20: 1242. 1887; 23: 3186. 1890; 24: 3834 and 3836. 1891.

Rubber is chemically close to the terpenes and is a cyclic hydrocarbon. The so-called artificial rubber¹ is formed by the condensation of isoprene $\text{CH}_2=\text{CH}-\text{C}(\text{CH}_3)=\text{CH}_2$ by the action of concentrated acetic acid in a fusion tube at 100° . According to Ostromyslensky,² rubber is easily obtained from β -myrcene, an aliphatic terpene which occurs in many plants and which can also be synthesized from isoprene.



"Artificial rubber" is obtained by warming β -myrcene with sodium and benzoyl peroxide.

Most investigators ascribe to rubber a very high molecular weight. According to Harries,³ the grouping $=\text{C}(\text{CH}_3)-\text{CH}_2-\text{CH}_2-\text{CH}=\text{}$ is very important in rubber. Rubber very likely contains dimethyl-cyclo-octadiene complexes.



I, 5-dimethyl-cyclo-octadiene

Ostromyslensky believes that rubber is a condensation product of β -myrcene. On the basis of various considerations, he concludes that the molecular weight of rubber is not so high as other writers suppose.

Gutta-percha is very close to rubber. This important substance is contained in the latex of a few *Sapotaceae*, *Asclepiadaceae* and *Apocynaceae*. Harries believes that gutta-percha is a stereo-isomeric form of rubber.

The formation of rubber in plants seems to be a process closely related to the formation of terpenes. It is impossible to consider the method of formation of rubber in plants more carefully because the immediate parent substance of rubber, the polymerization of which gives the natural product, is unknown.

8. HYDROAROMATIC COMPOUNDS

These substances are derivatives of hexahydrobenzene (hexamethylene) C_6H_{12} . They were mentioned in the consideration of the sugars because the

¹ Hoffman, F. Chem. Zeit. 36: 946. 1912; Harries, C. Gummi-Zeitschr. 24: 850. 1910; Idem. Ber. chem. Ges. 48: 863. 1915; Perkin, W. H. Jr. Jour. Soc. Chem. Ind. 31: 616. 1912.

² Ostromyslensky, A. Jour. russ. chem. Ges. 47: 1910, 1928, 1932 and 1941. 1915 (Russian). See also Luff. Jour. Soc. Chem. Ind. 35: 983. 1916.

³ Harries, C. Ber. chem. Ges. 38: 3985. 1905.

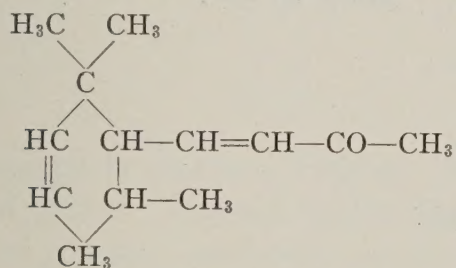
polyvalent hydroaromatic alcohols in living cells change surprisingly rapidly into sugars. The reverse process of conversion of sugars into cyclic polyvalent alcohols also appears to take place easily. The theoretical importance of this process is that some hydroaromatic substances readily change to phenol derivatives outside of living cells. In this way it is conceivable that the aromatic substances which are very common in plants are synthesized from sugar by way of the intermediate stage of the hydroaromatic compounds.

d-Quercite $C_6H_7(OH)_5$ (penta-hydroxy-hexa-hydro-benzene). Melts at 234° . $[\alpha]_D = +24.2^\circ$. Sweet prisms. Found first in acorns,¹ quercite also occurs in various other plants. Isomeric with quercite is polygalite from *Polygala amara*.²

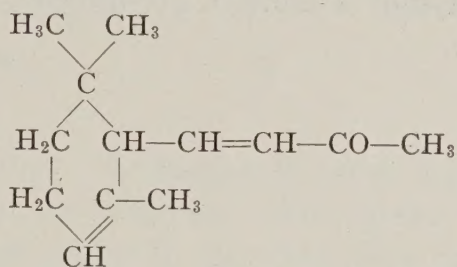
i-Inosite $C_6H_6(OH)_6$ ³ (hexa-hydroxy-hexa-hydro-benzene) is so much like the hexoses in its properties that for a time it was known as "cyclic sugar." Its empirical formula is identical with that of hexoses. Phytin, a hexa-phosphoric acid ester of inosite $C_6H_6-(O-H_2PO_3)_6$ is especially interesting (cf. p. 215).

The inosite ethers occurring in plants are: **bornesite**, the monomethyl ether; **dambonite** the dimethyl ether (both found in rubber); **pinitite**, the optically active methyl ether of d-inosite (in the cambium of conifers); and **quebrachite**, the optically active ether of l-inosite (in the latex of the rubber tree *Hevea brasiliensis*).

The hydroaromatic isomeric ketones **irone** and **ionone** are volatile oils. Irone is the cause of the fragrance of violets.⁴ It boils at 144° with 16 mm. Ionone has the same odor but it is a synthetic product prepared by the condensation of citral with acetone.⁵

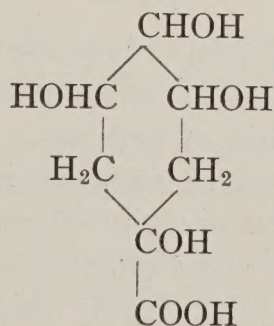


Irone



Ionone

Quinic acid (hexa-hydro-tetra-hydroxy-benzoic acid). Melts at 161.5° . $[\alpha]_D = -43.8^\circ$.



¹ Braconnot. Ann. de chim. et de physique (3) 27: 392. 1849.

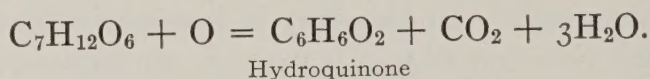
² Chodat, R. Ann. sci. phys. et nat., Genève, p. 590. 1888.

³ Maquenne, L. Compt. rend. 104: 1719. 1887; 109: 811. 1889; Maquenne et Tanret. *Ibid.* 110: 86. 1889.

⁴ Tiemann und Krüger. Ber. chem. Ges. 26: 2675. 1893.

⁵ Barbier et Bouveault. Bull. Soc. Chim. (3) 15: 1002. 1896; Tiemann. Ber. chem. Ges. 31: 808. 1898.

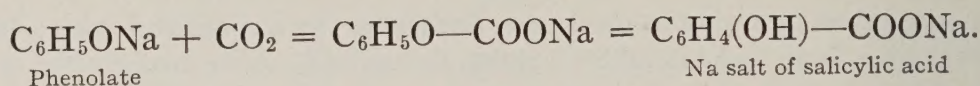
Is present in large quantities in coffee beans, in cinchona bark, in whortleberries and in many other plants. This interesting substance is a material from which microorganisms form sugar¹ and, on the other hand, readily changes into hydroquinone and protocatechuic acid.



According to the latest information, protocatechuic acid, pyrocatechol and hydroquinone are formed in cultures of the mold *Aspergillus niger* on quinic acid.² It is therefore allowable to assume that quinic acid is sometimes a transition stage from sugar to the benzene derivatives and especially the tannins.

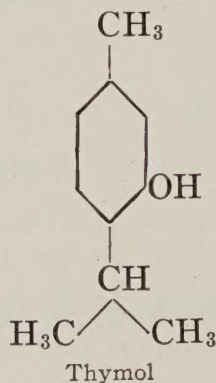
9. PHENOLS AND OTHER AROMATIC COMPOUNDS

Phenols have acid properties and form rather stable compounds with metals (phenolates). The phenol esters saponify easily. The phenolates have the capacity to add on CO_2 and to change it to a carboxyl group:



The phenol acids readily split off CO_2 . The polyvalent phenols are easily oxidized, especially in alkaline solution, and the benzene nucleus is rather easily opened for the addition of more hydroxyls.

Thymol (1-methyl, 4-isopropyl, 3-phenol). Melts at 50° .



Contained in various ethereal oils.³ May be an intermediate between the terpenes of the dipentene group and the aromatic substances. The side chains of thymol are identical with those of terpenes.

Carvacrol (1-methyl, 4-isopropyl, 2-phenol) is isomeric with thymol. Volatile. Boils at 236° . Also found in many ethereal oils.⁴

Pyrocatechol $\text{C}_6\text{H}_4(\text{OH})_2$ (1,2-dihydroxy-benzene). Melts at 105° . Reduces silver nitrate in the cold. Easily oxidized in alkaline solution with

¹ Kostytshew, S. Zeitschr. f. physiol. Chem. **111**: 236. 1920. [For the latest study of the rôle and importance of quinic acid in plants see Kiesel, A. Planta. **12**: 131-143. 1930.]

² Butkewitsch, W. Biochem. Zeitschr. **145**: 442. 1924.

³ Cf. Goulding, E. and Pelly. Proc. Chem. Soc. **24**: 63. 1908; Doveri, L. Ann. de chim. et de physique (3) **20**: 174. 1847; Rodié, J. Bull. Soc. Chim. de France (4) **1**: 492. 1907.

⁴ Umney, J. C. and C. T. Bennett. Pharm. Jou. (4) **21**: 860. 1905; Holmes, E. M. Ibid. **79**: 378. 1907; Pickles. Jour. Chem. Soc. (London) **93**: 862. 1908; and others.

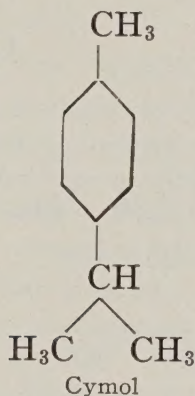
the formation of brown substances. Especially common in species of *Salix*. **Guaiacol** $C_6H_4(OH)-O-CH_3$, the methyl ester of pyrocatechol, with m.p. 31° , is still more easily oxidized.

Hydroquinone $C_6H_4(OH)_2$ (1, 4-dihydroxy-benzene). Melts at 172° . Oxidizes to red quinone. Hydroquinone is contained in the glucoside arbutin and is very common in the plant kingdom in this form.

Phloroglucin $C_6H_3(OH)_3$ (1, 3, 5-trihydroxy-benzene). Melts at 207° . Is contained in very many glucosides and tannins. Easily oxidized with a breaking of the ring. Various **fern acids** are to be regarded as derivatives of phloroglucin. They have no carboxyl groups and depend on the phenol group for their acid properties. In fern acids, as in all derivatives of phloroglucin, ketone groups are easily formed by intramolecular rearrangement or by change of the benzene ring to the hexamethylene ring.

Of the aromatic hydrocarbons, only

Cymol



is common in plants. Volatile. Boils at 175° . Contained in many ethereal oils of plants.¹ Cymol is closely related to the terpenes. Both cymol itself and its phenols, carvacrol and thymol, might easily be obtained from various terpenes.

The aromatic alcohols and ketones are common in various plant secretions, balsams and resins.

Benzyl alcohol $C_6H_5-CH_2OH$. Boils at 206° . Occurs as the acetic acid ester in ethereal oils of the jasmine flowers,² and as esters of benzoic acid and cinnamic acid in balsam of Peru and in other balsams.³

Cinnamyl alcohol (styron) $C_6H_5-CH=CH-CH_2OH$.⁴ Melts at 33° . As the ester of cinnamic acid, it forms the principal constituent of the valuable balsams from the bark of *Liquidambar styracifluum* and *L. orientale*; occurs also in the balsam of Peru.

Saligenin $C_6H_4OH-CH_2OH$ (2-hydroxy-benzyl alcohol). Melts at 82° . Contained as the glucoside salicin in the bark of various species of *Salix* and *Populus*.⁵

¹ Pickles, *loc. cit.*; Wakeman. Chem. Absts., p. 1170. 1912; Rodié. *loc. cit.*; and others.

² Hesse, A. Ber. chem. Ges. 32: 2611. 1899.

³ Kachler, J. Ber. chem. Ges. 2: 512. 1869; Busse, E. *Ibid.* 9: 830. 1876; and others.

⁴ Gössmann. Liebigs Ann. d. Chem. 99: 376. 1856.

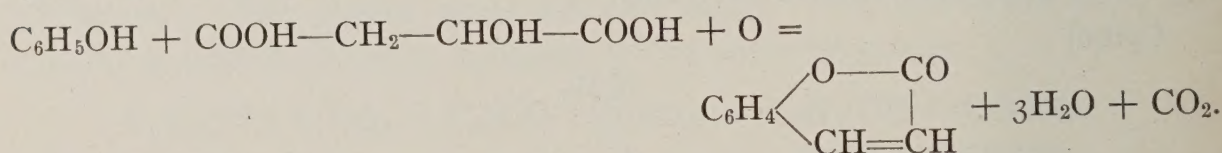
⁵ Leroux. Ann. de chim. et de physique (2) 43: 440. 1830; Bridel. Jour. Pharm. et Chim. (7) 19: 429. 1919; 20: 14. 1919; Irvine, J. C. and R. E. Rose. Proc. Chem. Soc. 22: 113. 1906; *Idem.* Jour. Chem. Soc. (London) 89: 814. 1906; and others.

Benzaldehyde C_6H_5-CHO . Boils at 179° . A highly refractive oil. Occurs in the very common glucoside amygdalin and also in the free condition as a cleavage product of this glucoside.¹

Cinnamaldehyde $C_6H_5-CH=CH-CHO$. Boils at 252° . An oil that is volatile in steam. Widely distributed in ethereal oils. Probably changes by the Cannizzaro reaction into cinnamic alcohol and cinnamic acid (see above).

Vanillin $C_6H_4(OH)(O-CH_3)-CHO$ (3-methyl ether of 3, 4-dihydroxy-benzaldehyde). Melts at 87° .² Common in various glucosides and contained in large quantities in the pods of *Vanilla planifolia*.

The aromatic carboxylic acids are contained in plants largely in the form of esters and lactones, but they also occur in the free condition. They are crystalline substances, not all of which are readily dissolved in water but they are all very soluble in alcohol and ether. The lactones of the unsaturated 1, 2-hydroxy acids, called cumarins, are noteworthy. These substances can be synthesized from phenols and malic acid:



Benzoic acid C_6H_5-COOH . Melts at 121° . Common in ethereal oils and resins as the ester of various alcohols.³ Slightly soluble in cold water.

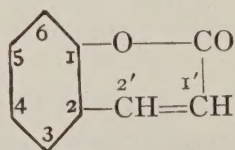
Cinnamic acid $C_6H_5-CH=CH-COOH$. Melts at 133° . In many balsams, also in leaves of various plants. The calcium salt is scarcely soluble in water.⁴

Salicylic acid $C_6H_4(OH)-COOH$ (2-hydroxy-benzoic acid). Melts at $155-156^\circ$. The salicylic salts of the monovalent metals are soluble in water but the salts of the divalent metals are for the most part very slightly soluble. Salicylic acid is very common in plants, chiefly in the form of esters and glucosides.⁵ It is readily separated from the aliphatic acids by chloroform.

Among the other very common acids, we can mention the ortho-hydroxy-cinnamic acids which occur partly in the free condition but mostly as cumarins (see above).

Cumaric acid $C_6H_4OH-CH=CH-COOH$ (2-hydroxy-cinnamic acid). Melts at $207-208^\circ$. Found mostly in the form of its lactone, the so-called coumarin, in various plants.⁶

Coumarin



is a fragrant, solid substance. Melts at 67° . Forms the principal constituent of the odorous substance of a large number of plants, in both Europe and the tropics, including *Anthoxanthum odoratum* and other grasses, *Asperula odorata*, ferns, and species of *Galium*, *Melilotus*, *Phoenix*, etc.

¹ Wahlbaum, H. Jour. f. prakt. Chem. 68: 325 and 424. 1903; Dodge. Jour. Ind. Eng. Chem. 10: 1005. 1918.

² Tiemann und Haarmann. Ber. chem. Ges. 7: 608. 1874; 8: 1175. 1875; Busse, Arb. d. Reichs-Gesundheitsamte. 15. I. 1899.

³ The first information by Mason, G. F. Jour. Amer. Chem. Soc. 27: 613. 1905.

⁴ Schering, K. Pharm. Week. 44: 984. 1907.

⁵ Griffiths, G. Chem. News. 60: 59. 1889; Power, F. B. and Salway. Amer. Jour. Pharm. 83: 1. 1911; and others.

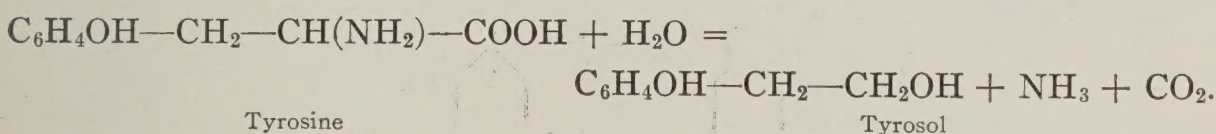
⁶ Schiff. Ber. chem. Ges. 5: 665. 1872; Tiemann und Herzfeld. Ibid. 10: 283. 1877; and others.

Caffeic acid $C_6H_3(OH)_2-CH=CH-COOH$ (3, 4-dihydroxy-cinnamic acid). Melts at 195° . Is a constituent of the tannin known as caffetannic acid and unites with quinic acid to form the so-called chlorogenic acid (see below).

Cumarin derivatives are very common in plants but of them we can mention only **daphnetin** (3, 4-dihydroxy cumarin) and **esculetin** (4, 5-dihydroxy cumarin). The former occurs as the glucoside daphnin in some species of *Daphne*; the latter is contained in many plants as the glucoside esculin.

The problem of the formation of the aromatic substances in plants is still unsolved. The great variety of benzene derivatives in plants is surprising; in the animals there are not so many aromatic substances. It may therefore be assumed that the benzene derivatives are formed from the surplus of sugars formed in the light, which have not been utilized for the general purposes of structural or operating metabolism. It is conceivable that the sugars may change to aromatic compounds by way of terpenes¹ or by way of hydroaromatic compounds. The possible transition of sugars into these substances was indicated above. As for the processes of dehydration, which lead from the terpenes or hexamethylene derivatives to the phenol derivatives, such reactions proceed smoothly in living plant cells.

Aromatic amino acids might also be produced by the removal of the side chains or by deaminization of various aromatic alcohols and acids. Thus tyrosine is deaminized as follows,² according to F. Ehrlich (p. 309):



Of course, it must be borne in mind that the carbon frameworks of the aromatic acids themselves are synthesized from sugar.

10. TANNINS

Tannins is the name given to a group of substances in plants, which have a harsh flavor, yield black precipitates with iron salts, form almost insoluble compounds with potassium bichromate, proteins and alkaloids and are oxidized to reddish-brown amorphous substances, the so-called phlobaphenes. Such a color reaction takes place, for example, at the cut surfaces of chopped apples and some other succulent fruits.³

The tannins so far investigated always contain phenol acids. They are very common in plants but usually occur in the cell sap rather than in the protoplasm. According to Freudenberg (*loc. cit.*), tannins are divided into two large groups. To the first group belong substances which can be hydrolyzed, especially esters and glucosides; in the second group are unhydrolyzable substances with complicated rings. The one type contain phloroglucin while

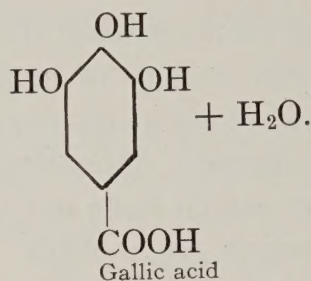
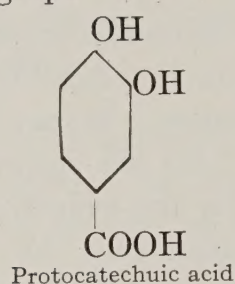
¹ For example, camphor changes to cymol under the influence of P_2O_5 . Cf. Dumas et Delalande. Ann. Chim. et Pharm. 38: 342. 1841. It is just as easy to form cymol from sabinol and some other terpenes. This is the usual method of preparing cymol.

² Ehrlich, F. Biochem. Zeitschr. 79: 232. 1917; cf. also Yukawa, M. Jour. Coll. Agric. Tokyo. 5: 291. 1915.

³ For the literature of tannins see: Perkin and Everest. The natural colouring matters. 1918; Fischer, E. Untersuchungen über Depside und Gerbstoffe. 1919; Freudenberg, K. Die Chemie der natürlichen Gerbstoffe. 1920; Lloyd, F. Jour. Amer. Leather-chem. Assoc. 17: 436. 1922.

the others are free from it. The following are the commonest components of tannins.

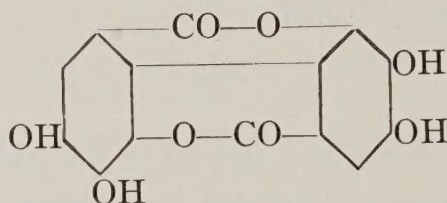
Protocatechuic acid (3,4-dihydroxy-benzoic acid). Melts at 194–195°. Reduces ammoniacal solutions of silver and yields a blue-green color with ferric chloride. Protocatechuic acid is a part of many tannins. It occurs free in the wine grape.¹



Gallic acid (3, 4, 5-trihydroxy-benzoic acid). Melts at 240°. Oxidizes readily in the air to form brown products. Is contained in many “depsides,”¹ *i.e.*, glucoside-like tannins which can be hydrolyzed.

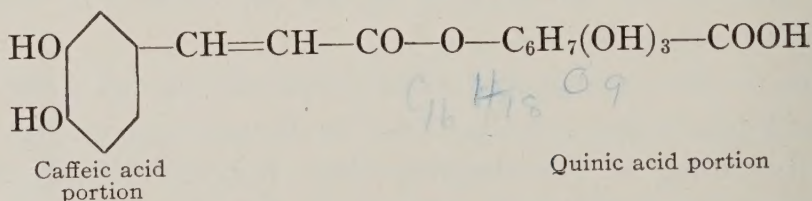
Tannic acid, a depside of uncertain constitution,² is typical of this group of tannins. It gives a number of characteristic color reactions³ and is hydrolyzed by the enzymes of some fungi with the formation of gallic acid.⁴ It is very common in plants.

Ellagic acid



Yellowish powder.⁵ Does not melt but sublimes and partly decomposes. Ellagic acid is formed from gallic acid by weak oxidation and when allowed to remain in alcoholic solution.⁶ This reaction shows how easily the condensation of the aromatic constituents of tannins takes place. Ellagic acid is contained in many tannins.

Chlorogenic acid



It has been found recently that the presence of this acid in pigmented onion scales is related to the resistance of the plant to disease. See Link, Angell and Walker. *Jour. Biol. Chem.* **81**: 369–375. 1929; Link, Dickson and Walker. *Ibid.* **84**: 719–725. 1929.—*Ed.*

¹ Fischer, E. *Ber. chem. Ges.* **51**: 1804. 1918; **52**: 7, 809 and 829. 1919; *Idem.* Untersuchungen über Depside und Gerbstoffe. 1919.

² Iljin. *Jour. russ. chem. Ges.* **45**: 157. 1914; Fischer, E. und K. Freudenberg. *Ber. chem. Ges.* **47**: 2485. 1914; and others.

³ Young, S. *Chem. News.* **48**: 31. 1883; Harnack. *Arch. d. Pharm.* **234**: 537. 1900; and others.

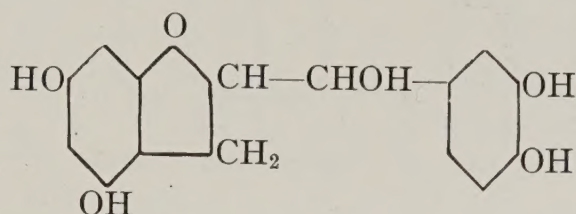
⁴ Fernbach. *Compt. rend.* **131**: 1214. 1900; Manéa, S. Thèse, Genève. 1904.

⁵ Graebe, C. *Ber. chem. Ges.* **36**: 212. 1903; and others.

⁶ Freudenberg, K. *Ber. chem. Ges.* **53**: 232. 1920; *cf. also* Keegan. *Chem. News.* **110**: 211. 1914; **113**: 85. 1916.

is a depside¹ which is split by the same enzymes which hydrolyze tannic acid. Chlorogenic acid is a constituent of caffetannic acid and occurs in this form in coffee beans, in seeds of *Strychnos*, etc.

Catechin



the constitution of which is represented by the accompanying new formula by Freudenberg,² is a valuable substance. It is common in some expensive tropical woods. Very soluble in alcohol but barely soluble in water.

The origin of tannins in plants is to be traced largely to the formation of aromatic compounds. We are therefore quite justified in supposing that sugars are the parent substances of the tannins. Much more is formed in the light than in the shade.³

The nutritive value of the benzene ring for microorganisms is very low and it may be assumed that tannins, phenols and other aromatic substances are not of food value in the higher plants either. The ease with which the phenol groups are oxidized makes it probable that the aromatic substances function as acceptors in respiration and in the case of other physiological oxidations. According to Palladin's views,⁴ tannins and glucosides are peculiar reserve substances which yield easily oxidized chromogens on hydrolysis. According to Palladin, these chromogens play an important rôle in plant respiration (*cf.* Chap. VIII).

However, this is not the only way in which tannins are important. They seem to be important in changes in permeability of the plasma membrane. There are interesting but poorly investigated transformations of tannins in cork. Tannins may also take part in the formation of wood. Some of the earlier writers thought that tannins are by-products but this is very unlikely.

II. NITROGEN-FREE SUBSTANCES WITH COMBINED RINGS

These are various plant pigments which are derivatives of anthracene, xanthone and flavone. In some plants they occur free but are mostly compounds of various glucosides. In this form many easily oxidized pigments are held previous to oxidation.

The anthracene molecule is a combination of three benzene rings. Anthraquinone is a para-diketone of anthracene and its derivatives are especially common in plants. Xanthone

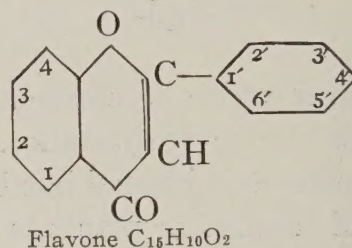
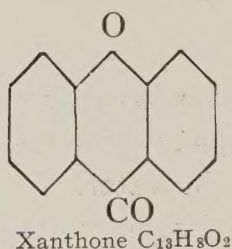
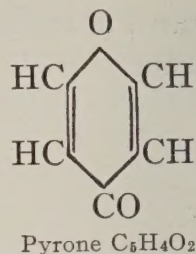
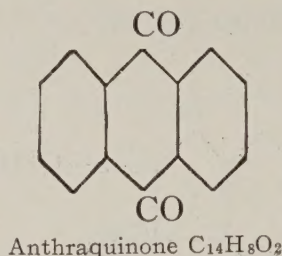
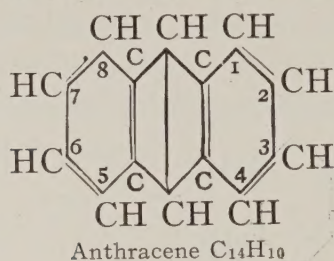
¹ Freudenberg, K. und Vollbrecht. Zeitschr. f. physiol. Chem. 116: 277. 1921.

² Freudenberg. Ber. chem. Ges. 53: 1416. 1920. The position of the single secondary hydroxyl group (not the phenol groups!) is still uncertain.

³ Kraus, Gr. Sitzungsber. d. Naturforscher, Halle. 1884; Henry. Ann. Soc. Agron. 1887; Büsgen, M. Beobachtungen über das Verhalten der Gerbstoffe. 1889; Daniel. Rev. gén. bot. 2: 391. 1890; Goris. Compt. rend. 136: 902. 1903; and others.

⁴ Palladin. W. Ber. bot. Ges. 26a: 125, 378 and 379. 1908; 27: 101. 1909; 29: 472. 1911; 30: 104. 1912; Idem. Biochem. Zeitschr. 27: 442. 1910; Idem. Rev. gén. bot. 23: 225. 1911; Idem. Zeitschr. f. Gärungsphysiol. 1: 91. 1912; Palladin und Lwow. Ibid. 2: 326. 1913; and others.

and flavone are products of a condensation of the benzene ring with the heterocyclic pyrone ring. The constitution of all these substances is made clear by the following formulae:



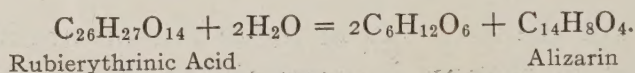
The formation of anthraquinone in plants is not understood. The most likely hypothesis is that two benzene rings combine by means of side chains. Among the many pigments of the anthraquinone group, the following may be named.

Chrysophanic acid $C_{14}H_8O_2(OH)_2-CH_3^1$ (1,8-dihydroxy-8-methyl-anthraquinone). Yellow flakes. Melts at 196° . In the roots of *Rheum*, *Rumex* and many other plants.

Emodin $C_{14}H_8O_2(OH)_3-CH_3^2$ (3, 6, 7-trihydroxy-2-methyl-anthraquinone). Orange-red needles. Melting point, 250° . In rhubarb roots and as glucosides in the roots of species of *Rhamnus*, of *Cascara sagrada* and other plants.

Aloins are colored derivatives of anthracene; probably of a glucoside nature; found in leaves of aloe. Are easily oxidized. Their constitution is unknown.³

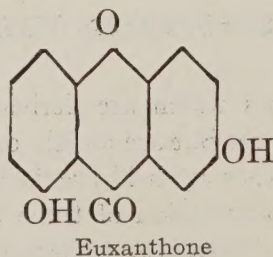
Alizarin $C_{14}H_6O_2(OH)_2^4$ (1, 2-dihydroxy-anthraquinone). Red needles which melt at 290° . Alizarin is the most important pigment of *Rubia tinctorum*, which contains a glucoside, rubierythrinic acid, which yields alizarin and glucose on hydrolysis:



Various other pigments of the anthracene group have been insufficiently investigated and offer interesting material for chemical studies.

A very interesting pigment of the xanthone group is

Euxanthone. Yellow crystals. Melts at 246° . Contained in leaves of *Mango* and other plants as glycuronide (euxanthinic acid).⁵



¹ Liebermann, C. und O. Fischer. Ber. chem. Ges. 8: 1102. 1875; Léger. Compt. rend. 154: 281. 1912; Idem. Jour. de phar. et de Chim. (7) 5: 281. 1912.

² Tutin, F. and Clewer. Proc. Chem. Soc. 25: 200. 1910; Idem. Jour. Chem. Soc. (London) 97: 1910; Fischer, O., Falko und Gross. Jour. f. prakt. Chem. 83: 208. 1911; 84: 369. 1911.

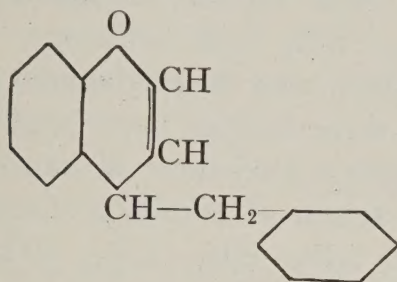
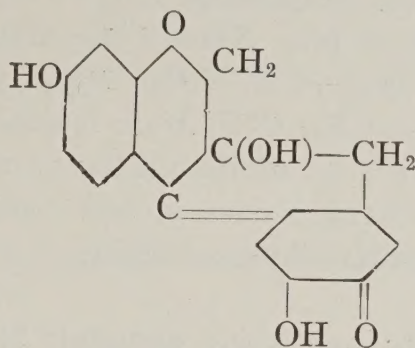
³ On this point see Léger. Jour. de phar. et de chim. (6) 25: 476 and 513. 1907.

⁴ Colin et Robiquet. Ann. de chim. et de physique (2) 34: 225. 1827; Graebe und Liebermann. Ber. chem. Ges. 2: 332. 1869.

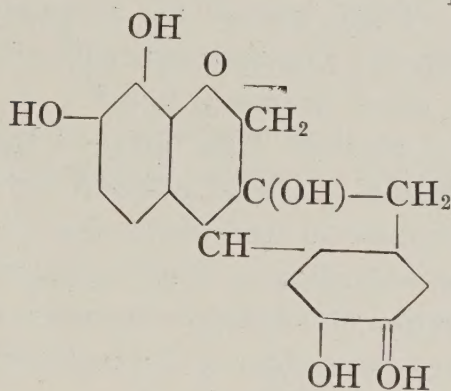
⁵ Stenhouse. Liebigs Ann. d. Chem. 51: 423. 1844; Ullmann, F. und L. Panchaud. Ibid. 350: 108. 1906.

The flavone derivatives which are extremely common in plants occur mostly as glucosides and were therefore considered in the preceding chapter.

The important pigments which can be prepared from the red and blue wood of various *Caesalpinaceae* are derivatives of benzylchromene or the so-called rufene:¹

Rufene²

Brasilein

Haematoxylin²

12. NITROGENOUS SUBSTANCES WHICH ARE CLOSELY RELATED TO THE CLEAVAGE PRODUCTS OF PROTEINS

In the preceding chapter it was pointed out that part of the amino acids and other cleavage products of protein are changed after the hydrolysis and are therefore used not to regenerate proteins but to form entirely different substances. The most prominent of these are the so-called "simple bases," *i.e.*, substances which are so closely related to the amino acids and purine and pyrimidine derivatives that their method of formation is quite evident. Further alteration of the simple bases leads to the formation of the alkaloids which often have a complicated constitution and are unlike the cleavage products of proteins.

It was pointed out in the preceding chapter that amino acids often split off CO_2 and change to the corresponding amines. This is the initial stage in the formation of the nitrogenous basic substances of plants.

Methylamine $\text{CH}_3\text{—NH}_2$ comes from glycocoll $\text{CH}_2(\text{NH}_2)\text{—COOH}$ by the removal of CO_2 . A solid substance thus becomes two gases, since methyl-

¹ Kostanecki und Lampe. Ber. chem. Ges. 35: 1667. 1902; Ballina, Kostanecki und Tambor. Ibid. 35: 1675. 1902; Kostanecki und Paul. Ibid. 35: 2608 and 4285. 1901; Kostanecki und Lloyd. Ibid. 36: 2193. 1903; Kostanecki und Rost. Ibid. 36: 2202. 1903.

² Perkin and Engels. Proc. Chem. Soc. 22: 132. 1906; Perkin and Robinson. Ibid. 22: 160. 1906; 23: 149. 1907; Idem. Jour. Chem. Soc. (London) 91: 1073. 1907; 93: 489 and 1115. 1908; 95: 381. 1909.

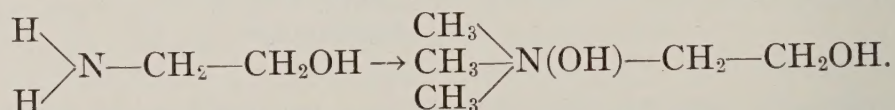
mine is a gas. Boils at -6.7° . Methylamine is formed by the bacterial putrefaction of protein and has also been found in the seed-plant *Mercurialis*.¹

The other simple amines are formed in the same way from the corresponding amino acids; **ethylamine** $\text{CH}_3\text{—CH}_2\text{—NH}_2$ from alanine, **tetramethylenediamine** (putrescine) $\text{NH}_2\text{—CH}_2\text{—CH}_2\text{—CH}_2\text{—CH}_2\text{—NH}_2$ from ornithine, **isobutylamine** $(\text{CH}_3)_2\text{—CH—CH}_2\text{—NH}_2$ from valine, and **isoamylamine** $(\text{CH}_3)_2\text{—CH—CH}_2\text{—CH}_2\text{—NH}_2$ from leucine. All these amines have been found in seed-plants.² In addition there is a whole series of analogous substances formed by the putrefaction of protein under the action of microorganisms. These include **pentamethylenediamine** (cadaverine) $\text{NH}_2\text{—CH}_2\text{—CH}_2\text{—CH}_2\text{—CH}_2\text{—CH}_2\text{—}$

$\text{CH}_2\text{—NH}_2$ from lysine, **agmatine** $\text{NH}_2\text{—}\overset{\text{NH}}{\parallel}\text{C—NH—CH}_2\text{—CH}_2\text{—CH}_2\text{—CH}_2\text{—}$ NH_2 from arginine, etc. These amines are probably also formed in seed-plants but are at once altered. The amines produced from amino acids might easily become fusel oils by direct deamination.³

Colamine (amino-ethyl alcohol) $\text{CH}_2(\text{NH}_2)\text{—CH}_2\text{OH}$ corresponds to the amino acid serine and occupies a special position insofar as a primary photosynthetic formation of colamine in seed-plants may be the intermediate stage in the synthesis of choline, according to Trier's statements.⁴

The further transformation of the simple amines is often only a methylation (cf. p. 346). Thus choline is a product of the methylation of colamine:



It was explained in the preceding chapter that under certain conditions the amino acids are also methylated. Thus **betaine** is formed from glycocoll and **stachydrine** from proline (p. 310). **Hypaphorine** is the betaine of tryptophane (p. 310).

In some cases both decarbonizing and methylation takes place at once. For example, this forms

Hordenine $\text{C}_6\text{H}_4\text{OH—CH}_2\text{—CH}_2\text{—N(CH}_3)_2$ ⁵ (dimethyl-para-hydroxy-phenyl-ethyl-amine) from tyrosine by the removal of CO_2 and the substitution of two methyl groups for the two hydrogen atoms attached to the nitrogen. It melts at 118° . In an analogous way from ornithine comes

Tetramethyl-putrescine $(\text{CH}_3)_2\text{N—CH}_2\text{—CH}_2\text{—CH}_2\text{—CH}_2\text{—N(CH}_3)_2$.⁶

Other nitrogenous bases are formed by complicated conversions of the amino acids but the connection between these two groups of substances is always

¹ Mörner, C. Th. Zeitschr. f. physiol. Chem. 22: 514. 1897; Hasebrock. Ibid. 12: 148. 1888; and others.

² Erdmann, E. & H. Ber. chem. Ges. 32: 1217. 1899; Ciamician e Ravenna. Arch. di fisiol. 9: 504; Idem. Atti d. Reale accad. dei Lincei, rendiconto (5) 20(I): 614; Thoms, H. und Thümen. Ber. chem. Ges. 44: 3325 and 3727. 1911.

³ Ehrlich, T. und P. Pistschimuka. Ber. chem. Ges. 45: 1006. 1912.

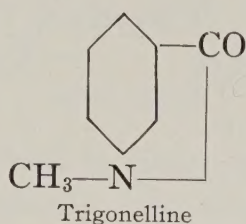
⁴ Trier, G. Zeitschr. f. physiol. Chem. 73: 383. 1911; 76: 496. 1912; 80: 409. 1912; Idem. Über einfache Basen. 1912.

⁵ Léger, E. Compt. rend. 142: 108. 1906; 143: 234 and 916. 1906; 144: 208. 1907.

⁶ Willsättter, R. und Heubner. Ber. chem. Ges. 40: 3869. 1907.

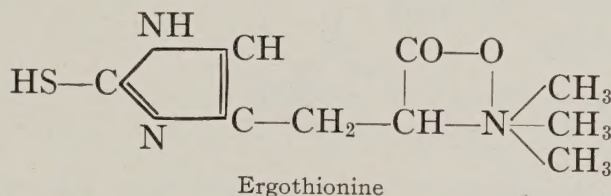
evident. For example, **muscarine**,¹ the poison of the fly fungus [*Amanita muscaria*], is both chemically and genetically related to choline and betaine. The constitution of muscarine has not been determined.²

Trigonelline



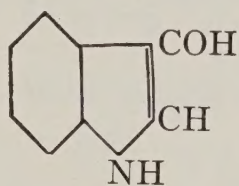
is the only simple pyridine base common in plants.³

Ergothionine



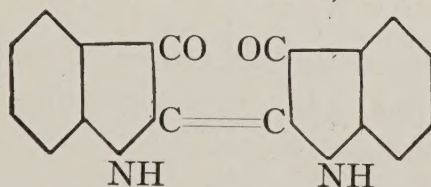
is the histidine betaine containing sulphur.⁴ Its formation from histidine can scarcely be doubted.

Indoxyl



is related to tryptophane and occurs in the so-called indigo plants as the glucoside indican (in union with one molecule of glucose). Bright yellow crystals. Melts at 85°. After cleavage of the glucoside, indican is oxidized in the air⁵ and yields the practically blue pigment, the so-called indigo. Indigo plants are those rich in indican or isatine (*Indigofera tinctoria*, *Polygonum tinctorium*, *Nerium tinctorium* and many others). Other plants contain isatine (*Isatis tinctoria* and others). Palladin (see p. 355) believes that indoxyl is a respiratory prochromogen.

Indigo (indigotine) is formed from two molecules of indoxyl by the removal of four atoms of hydrogen in the process of oxidation.⁶ It is a blue pigment with a red metallic sheen. Sublimes as reddish-violet fumes. Decomposes at 390–392°. Insoluble in water, alcohol or ether; in chloroform, oils, oil of turpen-



¹ Fischer, E. Ber. chem. Ges. 27: 166. 1894.

² On this point see Küng. Zeitschr. f. physiol. Chem. 91: 241. 1914; and others.

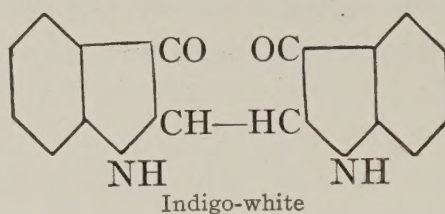
³ Jahns, E. Ber. chem. Ges. 18: 2518. 1885; Schulze, E. Zeitschr. f. physiol. Chem. 47: 507. 1906; 60: 155. 1909; 67: 52. 1910; Idem. Landwirt. Versuchs-Stationen 59: 340. 1904.

⁴ Tanret. Compt. rend. 149: 222. 1909; Barger and Evans. Trans. Chem. Soc. 99: 2336. 1911.

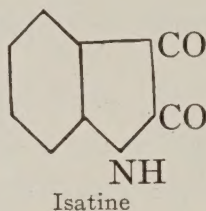
⁵ Nencki. Ber. chem. Ges. 9: 299. 1876; Bréaudat. Compt. rend. 127: 769. 1898; 128: 1478. 1899; and others.

⁶ Nencki. loc. cit. The first product of the oxidation of indoxyl is really not indigo but another substance.

tine and paraffin. Changes to the leuco compound, the so-called **indigo-white**¹

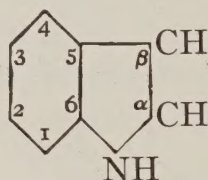


on reduction, by taking on two atoms of hydrogen.² **Isatine** is obtained by the oxidation of indigo with nitric acid. Indigo was once obtained only from



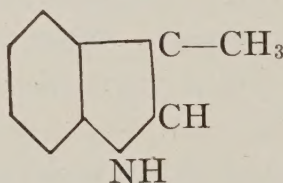
indigo plants. Now the pigment is synthesized³ and only the synthetic product is on the market.

Indol



the parent substance of all the products allied to tryptophane, was first found in jasmine flowers⁴ and in a few ethereal oils. On the whole, it is very common in plants. The flowers of *Robinia Pseudacacia* give a good yield of indol.⁵ Colorless. Melts at 52°.

Scatol (β -methyl indol)



Melts at 95°. Found in the wood of a few trees.⁶ Slightly soluble in water. Usually produced by the putrefaction of protein.

Simple bases of an alkaloid nature are formed in plants by simple methylation of the oxidized purine bases. The purine derivatives of the nucleic acids—adenine and guanine—are transformed by the action of specific oxidizing

¹ Dumas. Ann. de chim. et de pharm. 48: 257. 1843; Baeyer. Ber. chem. Ges. 15: 54. 1882.

² Baeyer. Ber. chem. Ges. 11: 1228. 1878.

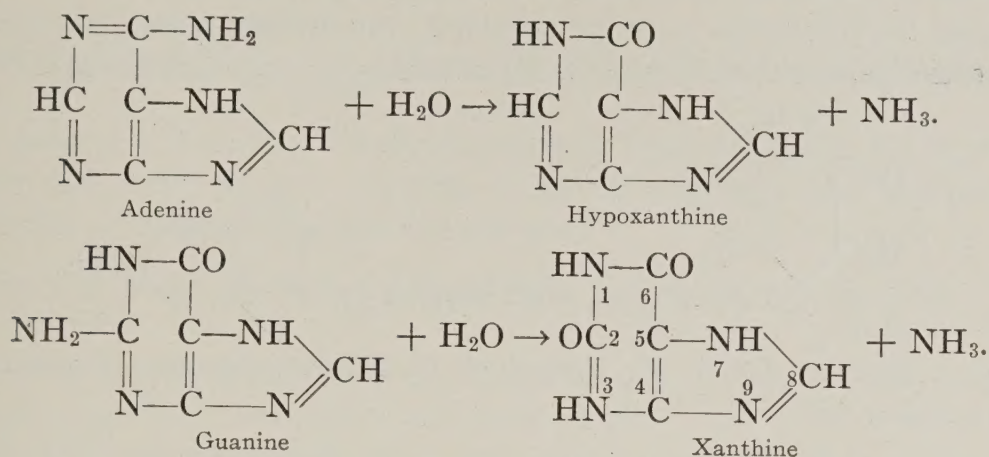
³ Baeyer und Emmerling. Ber. chem. Ges. 3: 515. 1870; Baeyer. Ibid. 13: 2254. 1880; Baeyer und Drewsen. Ibid. 15: 2856. 1882.

⁴ Soden, H. v. Jour. f. prakt. Chem. (2) 69: 256. 1904.

⁵ Elze, F. Chem. Zeit. 34: 814. 1910; and others.

⁶ Dunstan, R. Proc. Roy. Soc. London. 46: 211. 1890; Idem. Chem. News. 59: 291. 1889; and others.

enzymes, the so-called xanthoxidases, into hypoxanthine and xanthine, with the removal of NH_3 .¹



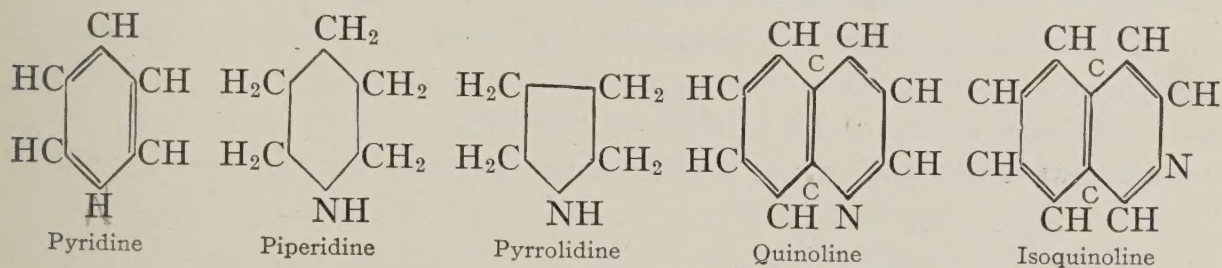
Theobromine (3, 7-dimethyl-xanthine) is the active substance of the cacao bean (up to 4%).² Very slightly soluble in water; does not melt but sublimes at $290-295^\circ$.

Theophylline (1,3-dimethyl-xanthine) is isomeric with theobromine. In tea leaves.³ Melts at 264° .

Caffeine (1, 3, 7-trimethyl-xanthine) is the active principle of tea and coffee.⁴ Melts at 235° .

13. ALKALOIDS

These are nitrogenous bases which have poisonous properties and are characteristic of different groups of plants.^k The configuration of the alkaloids can be very varied. Oftentimes very different kinds of nitrogenous basic substances, including the simple bases just described, are included in the term alkaloids. The characteristic alkaloids, however, are heterocyclic substances in which the nitrogen is a part of the ring. For example, the following rings are especially characteristic of alkaloids:



Most alkaloids contain oxygen. In some alkaloids, the only side chain united with the nitrogen is the methyl group CH_3 . The nitrogen can usually be removed only after complete destruction of the alkaloid molecule. Almost all

¹ Jones, W. und C. R. Austrian. *Zeitschr. f. physiol. Chem.* 48: 110. 1906; Winternitz und Jones. *Ibid.* 60: 180. 1909; Jones. *Ibid.* 65: 383. 1910.

² Wokressensky. *Jour. f. prakt. Chem.* 23: 394. 1841; *Idem.* *Liebigs Ann. d. Chem.* 41: 125. 1842; Fischer, E. *Synthesen in der Purin- und Zuckergruppe.* 1903.

³ Kossel, A. *Ber. chem. Ges.* 21: 2164. 1888; *Idem.* *Zeitschr. f. physiol. Chem.* 13: 298. 1888.

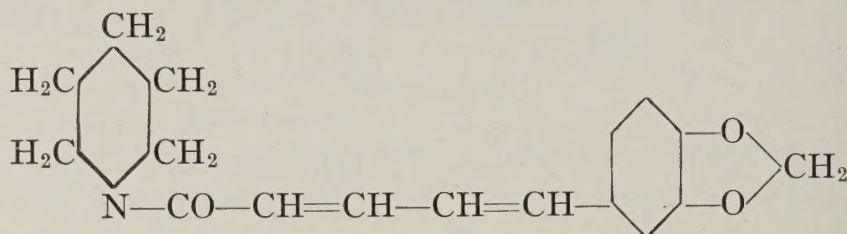
⁴ Pelletier. *Jour. de pharm.* (2) 244: Fischer, E. *loc. cit.*

^k For a complete and up-to-date monograph on these substances, see Henry, T. A. *The Plant Alkaloids.* 2nd. ed. 1924 (Blakiston).—*Ed.*

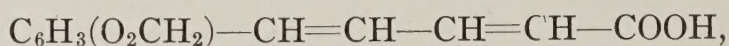
alkaloids are colorless. They exert a powerful toxic effect on animals. In plants, the alkaloids are generally united with various acids.

Among the numerous pyridine alkaloids, the following may be mentioned:

Piperine (piperidine-piperinic acid) accounts for the burning taste of species

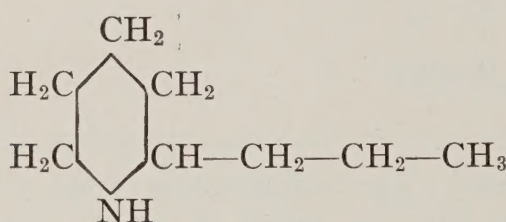


of *Piper*. Melts at 128° .¹ On hydrolysis by alkalis, piperine gives piperidine and piperinic acid,

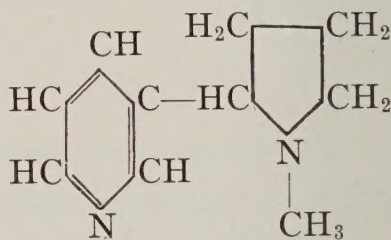


which is almost insoluble in water and the usual organic solvents. Melting point is 212° .

d-Coniine (d-propyl-piperidine) was found in *Conium maculatum* and a few other plants.² Synthesized long ago.³ Colorless liquid; boils at 166° ; $[\alpha]_D = +15.7^{\circ}$.



Coniine

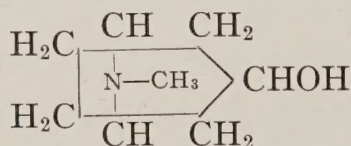


Nicotine

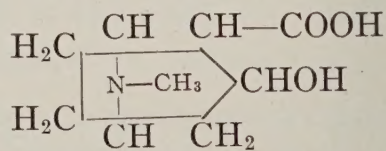
Nicotine (1-methyl-2- β -pyridyl-pyrrolidine). Linked with malic acid and citric acid in tobacco leaves. Synthetically prepared.⁴ Colorless oil. Oxidizes and forms a resin in the air. Boils at 246° with 730 mm. $[\alpha]_D = -166.39^{\circ}$. In tobacco leaves the nicotine content increases with age.⁵

The alkaloids which are especially important in pharmacology have a complicated constitution. Our knowledge of the chemical nature of some of the substances in this group is still very deficient.

Atropine and **cocaine** are esters of the alcohols tropine and ecgonine:⁶



Tropine



Ecgonine

¹ Oerstedt. Schweigg. Jour. 29: 80. 1920; Königs. Ber. chem. Ges. 12: 2341. 1879; 14: 1856. 1881; Fittig. Liebigs Ann. d. Chem. 152: 35 and 56. 1869; 159: 129. 1871; 168: 94. 1873; 216: 171. 1883; 227: 31. 1885.

² Farr and Wright. Pharm. Jour. 18: 185. 1904; Lepage. Jour. de chim. et de pharm. (5) 11: 10. 1885.

³ Hoffmann, A. W. Ber. chem. Ges. 14: 705. 1881; 15: 2313. 1882; 16: 558. 1883; 17: 825. 1884; 18: 5 and 109. 1885; Ladenburg, A. Ibid. 19: 439 and 2578. 1886; 39: 2486. 1906; 40: 3734. 1907.

⁴ Pictet, A. et A. Rotschy. Compt. rend. 137: 860. 1904; Idem. Ber. chem. Ges. 37: 1225. 1904.

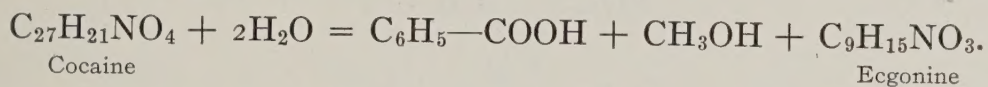
⁵ Mothes, K. Planta. 5: 563. 1928.

⁶ Willstätter, R. Liebigs Ann. d. Chem. 317: 307. 1901; Idem. Ber. chem. Ges. 30: 2679. 1897; 31: 1534 and 2498. 1898; Willstätter, R. und Müller. Ibid. 31: 1212 and 2655. 1898.

Atropine, a powerful poison, occurs in some *Solanaceae*, e.g., *Atropa Belladonna* and *Datura stramonium*. On hydrolysis, atropine decomposes into tropine and

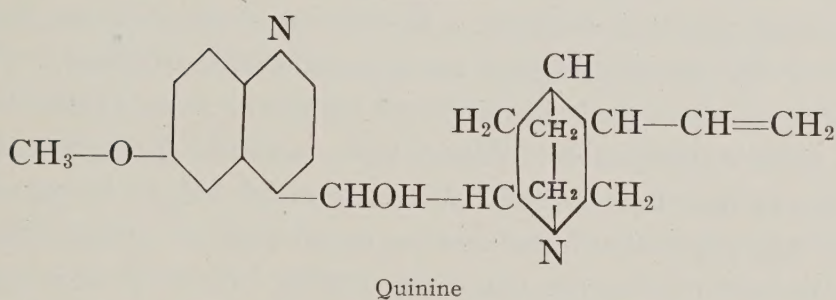
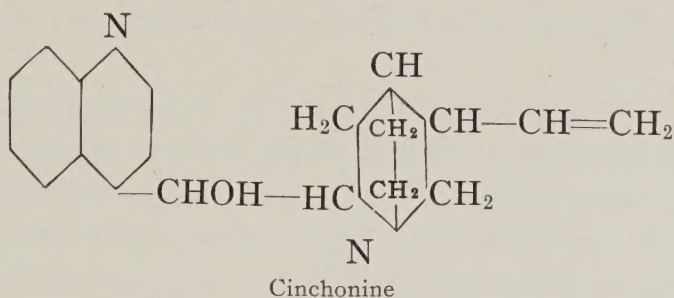
tropic acid $\text{C}_6\text{H}_5\text{—CH} \begin{cases} \text{COOH} \\ \text{CH}_2\text{OH} \end{cases}$ and is the ester of these two substances. The

melting point is $115\text{--}116^\circ$. Tropine is a strong base, soluble in water, alcohol and ether and melts at 63° . Cocaine is an ethereal ester-like compound of ecgonine with methyl alcohol and benzoic acid:¹



It is found in leaves of *Erythroxylon Coca* and other species of *Erythroxylon*. Has strong anaesthetic properties and melts at 98° .

The alkaloids of cinchona bark are united to quinic acid and have the following configurations:³



Cinchonine is very slightly soluble in water. Melts at 225° .⁴

Quinine is strongly levorotatory. Melts at 177° . The salts of quinine are fluorescent in solution.⁵

The isoquinoline ring is contained in some alkaloids of opium. The most important substance of this group is **morphine**, the constitution of which is not yet finally settled but it is probably represented by the accompanying formula.⁶ Melting point 247° ; rotates strongly to the right. **Codeine** and **thebaine** are very close to morphine. In codeine, the phenol group of morphine is linked as an ester with methyl alcohol.

¹ Lossen, W. Liebigs Ann. d. Chem. 133: 351. 1861.

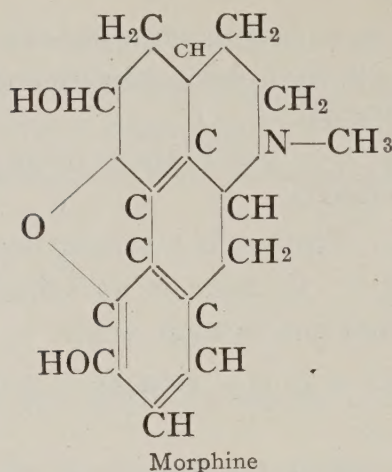
² Niemann. Liebigs Ann. d. Chem. 114: 213. 1860; Pozzi-Escot. Rev. gén. chim. pure et appl. 16: 225. 1913.

³ Königs. Ber. chem. Ges. 27: 900 and 1501. 1894; 28: 1986 and 3150. 1895; 30: 1328. 1897; 35: 1349. 1902; 37: 3244. 1904; Idem. Liebigs Ann. d. Chem. 347: 143. 1906.

⁴ Gerhard. Liebigs Ann. d. Chem. 44: 279. 1842; Butlerow und Wyschnegradsky. Ber. chem. Ges. 11: 1253. 1878.

⁵ Déniges, G. Bull. Soc. Pharm. Bordeaux. 49: 385. 1911.

⁶ Pschorr, R. Ber. chem. Ges. 33: 1810. 1900; 35: 4412. 1902; 37: 1926. 1904; 39: 3124. 1906; Braun und Aust. Ibid. 50: 43. 1917; Kaufmann und Dürst. Ibid. 50: 1630. 1917.



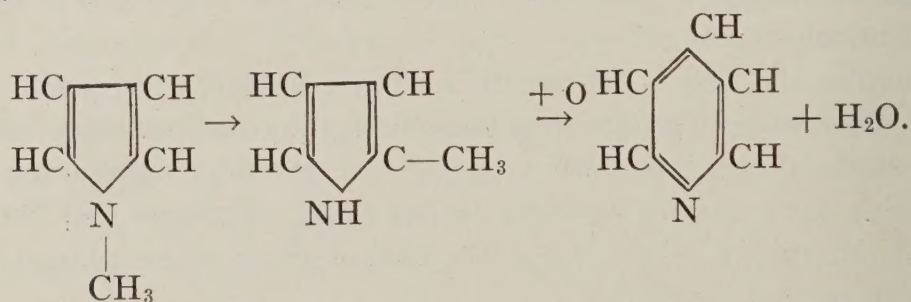
Important alkaloids of unknown constitution are:

Strychnine $C_{21}H_{22}N_2O_2$ from *Strychnos nux-vomica*.¹ Levorotatory. Melting point 269° . Very slightly soluble in water.

Brucine $C_{23}H_{26}N_2O_4$ accompanies strychnine and is probably its dimethyl ether.¹

The number of alkaloids found in plants is very great. It is interesting that certain alkaloids occur in certain plant families or genera, and could therefore be used as an exact character for classification. Many alkaloids have been found especially in the *Rubiaceae*, *Apocynaceae*, *Solanaceae*, *Papaveraceae* and *Leguminosae*. Plants which are rich in terpenes contain either no alkaloids or only very small amounts of them. In the case of plants containing alkaloids, the young, actively growing organs are especially rich in them.

Pictet² tries to show that alkaloids are produced from cyclic cleavage products of the protein molecules. There have actually been found in different plants volatile pyrrol and pyrrolidine bases which might be transition stages from the cleavage products of proteins to the alkaloids.³ Other likely transition stages have been mentioned on preceding pages. Pictet found that the pyrrol ring can be changed to the pyridine ring by methylation and subsequent oxidation:



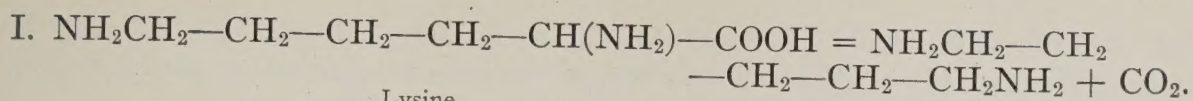
Pyridine can also be formed from some aliphatic compounds. Thus lysine changes to pentamethylene diamine with the removal of CO_2 and NH_3 and the latter passes to piperidine and on to the pyridine ring.⁴

¹ Pelletier et Caventou. Ann. de chim. et de physique (2) 10: 142. 1819; 12: 113. 1819; and others.

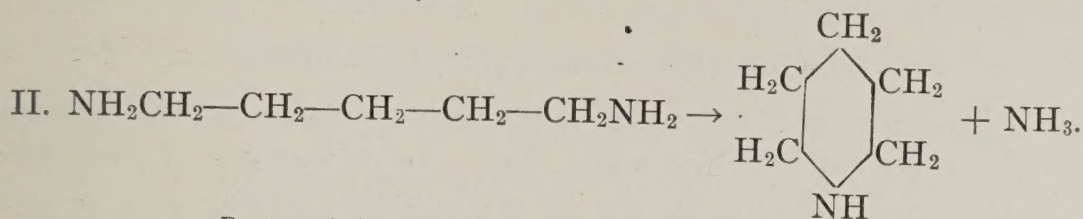
² Pictet, A. Ann. Sci. Phys. et Nat. Genève (4) 19: 139. 1905; Idem. Ber. chem. Ges. 38: 1946. 1905.

³ Pictet, A. et G. Court. Bull. Soc. Chim. de France (4) 1: 1001. 1907; Idem. Ber. chem. Ges. 40: 3771. 1907; Pictet. Arch. de pharmacie. 244: 384. 1906.

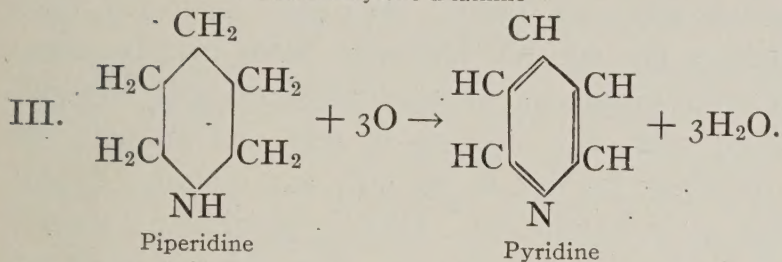
⁴ Cf. especially Robinson. Jour. Chem. Soc. (London) 111: 876. 1917.



Lysine



Pentamethylene Diamine



Piperidine

Pyridine

In an analogous way, pyrrolidine and then pyrrol is formed from tetramethylene diamine.

That the individual alkaloids are to be found only in a limited number of plants may depend on the fact that the formation of different alkaloids involves complicated material transformations. It is clear that the substances formed from protein cleavage products by simple and widespread reactions, must be very common. For example, if one is dealing simply with methylation, it is to be expected that the products of such a common chemical process are to be found in different kinds of plants. Actually caffeine, betaine and analogous substances related to the protein cleavage products are found in numerous plants of very different families. The alkaloids of the cinchonine or morphine group, on the contrary, are met with only in a few genera of a single family.

At present it is impossible to give a good definition of the physiological rôle of alkaloids. It is known that the alkaloid content in plants bearing them undergoes various fluctuations. Periods of consumption of alkaloid follow periods of its accumulation.¹ The rather widespread view that alkaloids are by-products is not based on experimental facts. Still less probable is the teleological explanation according to which alkaloids function as protective poisons. A whole series of reliable observations shows that *Cuscuta* and other parasitic plants penetrate host plants rich in alkaloids.² Birds and insects are often immune to various plant poisons.³ The idea has developed recently that alkaloids are active in living cells as stimulants and hormones.⁴ The significance of alkaloids may possibly become clearer when greater progress has been made in the chemistry of enzymes and vitamins.

Analytical Methods.—The alkaloids are usually precipitated from solutions by phosphomolybdic acid or a solution of bismuth iodide. Nitrophenols and picronic acid are also well suited to this purpose. The latter is also useful for quantitative determinations. The isolation of alkaloids in pure form usually meets with no serious difficulties because these substances

¹ Clautriau. Bull. Soc. Belge Micros. Vol. 18. 1894; Torquati. Arch. di farmcol. sperim. e scienze aff. 10: 62. 1911.

² d'Ippolito, G. Stat. sperim. agr. Ital. 46: 540. 1913; and others.

³ van Leersum, B. P. Pharm. Week. 46: 369. 1909; and others.

⁴ Ciamician e Ravenna. Sul signif. biol. degli alcaloidi nelle piante. 1921.

are slightly soluble in cold water and alcohol and crystallize out well. For the methods of preparation and quantitative determination of the individual alkaloids, reference must be made to the special literature.¹

14. VITAMINS AND ANALOGOUS SUBSTANCES

Recent discoveries have been made of interesting substances, the chemical nature of which has not yet been made clear. Small amounts of these substances are absolutely essential for the life of the higher animals and the lack of them results in certain diseases and even death. As Funk² suggested, these substances are called vitamins—a not entirely fortunate name because many vitamins not only contain no amine groups but are generally lacking in nitrogen.

Clinical studies have proved that some diseases of man and animals, the etiology of which was once puzzling, are due to the lack of a certain vitamin. These diseases are designated as avitaminoses. Direct experiments showed that animals are unable to synthesize the vitamins. They depend on plants for vitamins as they do for proteins.³ Hence, the physiological rôle of the vitamins is established by means of experiments on animals, the food of which contains all other substances necessary to maintain life, but which is free from a certain vitamin. Such experiments cannot be carried out with plants because they synthesize vitamins but there are various facts which support the idea that vitamins are also indispensable for the life of plants.

The general indications of an avitaminosis are first, that growth stops and the patient loses weight, a condition which is often regarded as due to lack of blood.⁴ In the second place, the functions of the organs of reproduction are reduced if the lack of vitamins is only slight.⁵ Finally, it is to be emphasized that avitaminosis *increases* the resistance of the organism against the toxic action of alkaloids.⁶ In addition, a lack of a given vitamin is shown by certain specific symptoms.

Two groups of vitamins are recognized. In the first one come Vitamin B and Vitamin C which are soluble in water. They are also soluble in ethyl and methyl alcohol but are insoluble in benzene, acetone, petrol ether, and other fat solvents.⁷ The second group includes the fat-soluble vitamins A, D and E which are also soluble in the foregoing organic solvents. These substances are practically insoluble in water.⁸

Vitamin A is contained in the green parts of plants. Its occurrence correlates with that of chlorophyll but it is not a constant factor in a given plant

¹ For example see Grafe, V. Handbuch d. biochem. Arbeitsmethoden by Abderhalden. 6: 108. 1912.

² Funk. Jour. Physiol. 43: 395. 1911; 44: 50. 1912.

³ Cf. however Leigh-Clare. Biochem. Jour. 21: 368. 1927; Bills, C. E. Jour. Biol. Chem. 72: 751. 1927; Lepkovsky and Nelson. Ibid. 59: 91. 1924; Parson and Hutton. Ibid. 59: 97. 1924; Parson and Reynolds. Ibid. 59: 731. 1924; and others.

⁴ Drummond and Marrian. Biochem. Jour. 20: 1229. 1926; Kon and Drummond. Ibid. 21: 632. 1927; Marrian, Baker, Drummond and Woolard. Ibid. 21: 1336. 1927; Bezssonoff. Bull. Soc. Chim. Biol. 10: 1179. 1928; 10: 1199. 1928.

⁵ Sure, B. Jour. Biol. Chem. 74: 37, 45, 55 and 71. 1927; 76: 673. 1928; Macy, Outhouse, Long and Graham. Ibid. 73: 153. 1927; Evans and Burr. Ibid. 76: 263. 1928.

⁶ Saiki, T. Ann. Inst. Pasteur. 48: 668. 1928.

⁷ Hart, Steenbock and Lepkovsky. Jour. Biol. Chem. 52: 241. 1922; Williams and Waterman. Ibid. 68: 499. 1926.

⁸ McCallum, Simmonds and Steenbock. Ibid. Vol. 29. 1917.

during different years.¹ The connection of vitamin A with carotin and lycopin is very striking; considerable amounts of vitamin A are contained in parts of plants which are without chlorophyll but which are rich in carotin or lycopin as, for example, in the roots of carrots, in fruits of tomato, etc. In plants and parts of plants which contain no pigments of the carotinoid group, vitamin A has so far never been found. Avitaminosis A manifests itself by means of the foregoing general symptoms and in addition by a degeneration of the mucous membranes which expresses itself in dangerous eye diseases and in diseases of the respiratory passages.²

Vitamin A is a light-sensitive substance³ which is also rapidly destroyed by the action of oxidizing and reducing agents. The properties of this substance correspond to those of an unsaponifiable lipid. Vitamin A is then also free from nitrogen; its molecular weight is less than 400.⁴ According to Euler, vitamin A is a carotinoid.⁵ In any case, it can hardly be doubted that there is a double bond in its molecule.

Vitamin B appears to be a collective term⁶ which includes a whole group of vitamins. Of the component parts, vitamins B₁ and B₂ are especially well known. The former is rapidly destroyed by a rise in temperature and is also unstable towards alkalis. A deficiency of vitamin B₁ initiates the so-called polyneuritis in birds and paralytic phenomena in mammals, in addition to the general symptoms mentioned above. Vitamin B₂ is scarcely affected by alkalis at 120°. The specific disturbance with avitaminosis B₂ is a severe disease of the skin.⁷ It is possible that vitamin B₂ represents a combination of different substances.⁸ Vitamin B (most of which is B₁) is contained in seed-coats and embryos of cereals. In yeast cells and in some molds there are also large quantities of vitamin B (largely B₂). It is to be emphasized that of all the vitamins, only vitamin B occurs in plants without chlorophyll. However, in green plants most of the vitamin B is to be found in organs which contain chlorophyll.⁹

Vitamin B is a nitrogen-free substance¹⁰ which has a high capacity for adsorption and is precipitated by lead acetate.¹¹ Vitamin B (chiefly B₁) is

¹ Moore. *Biochem. Jour.* 21: 870. 1927; Coward. *Jour. Biol. Chem.* 72: 781. 1927; Heller. *Ibid.* 76: 499. 1928; Crist and Dye. *Ibid.* 81: 525. 1929; McLaughlin. *Ibid.* 84: 525. 1929.

² Emmet and Peacock. *Jour. Biol. Chem.* 56: 679. 1923; Bethke, Kennard and Sassaman. *Ibid.* 72: 695. 1927; Steenbock and Coward. *Ibid.* 72: 765. 1927; Macy, Outhouse, Long and Graham. *Ibid.* 73: 153 and 175. 1927.

³ Willmot, Moore and Wokes. *Biochem. Jour.* 20: 1292. 1926; Collison, Hume, Smedley-McLean and Smith. *Ibid.* 23: 634. 1929; Bezssonoff. *Rev. Path. Végét.* 14: 142. 1927.

⁴ Drummond, Channon and Coward. *Biochem. Jour.* 19: 1047. 1925; Drummond and Baker. *Ibid.* 23: 274. 1929.

⁵ Euler, B. v., H. v. Euler und Hellstrom. *Biochem. Zeitschr.* 203: 379. 1928; Euler, Euler and Karrer. *Helv. Chim. Acta.* 12: 278. 1929; Moore. *Biochem. Jour.* 23: 803. 1929.

⁶ Chick and Roscoe. *Biochem. Jour.* 22: 790. 1928; 23: 498. 1929.

⁷ Goldberger, Wheeler, Lillie and Rogers. *U. S. Publ. Health Rept.* 41: 297. 1926.

⁸ Kennedy and Palmer. *Biochem. Jour.* 26: 591. 1928; Williams, Waterman and Gurin. *Jour. Biol. Chem.* 83: 321. 1929.

⁹ Chick and Hume. *Proc. Roy. Soc. London.* 90: 44 and 60. 1917; Osborne and Mendel. *Jour. Biol. Chem.* 37: 189. 1919; Plimmer, Rosedale, Raymond and Lowndes. *Ibid.* 21: 1241. 1927.

¹⁰ Jansen and Donath. *Proc. Kon. Akad. Wet. Amsterdam.* 29: 1390. 1926; *Chem. Week.* 23: 201. 1926; Seidell. *Jour. Biol. Chem.* 82: 639. 1929.

¹¹ Williams and Waterman. *Jour. Biol. Chem.* 78: 311. 1928; Kinersly and Reader. *Biochem. Jour.* 22: 276. 1928; Levene. *Jour. Biol. Chem.* 79: 465. 1928.

destroyed by boiling, even in neutral solution, with excess to air or in the autoclave at 120° with oxygen excluded.¹ Its molecular weight appears not to exceed 300.

Vitamin C is likewise water-soluble. Scurvy is nothing but avitaminosis C. This is perhaps the commonest of all avitaminoses. Chlorophyll-bearing parts of plants are especially rich in vitamin C² but still, unlike vitamin A, it is also present in some juicy fruits (lemons!) and underground containers of reserve substances which contain neither chlorophyll nor yellow carotinoids.³

Vitamin C, like vitamin B, seems to be a collective term. According to recent investigations, one component of vitamin C is thermo-labile but the others are thermo-stable.⁴ Vitamin C contains no nitrogen⁵ and has a molecular weight of about 200.⁶ It oxidizes very readily even by molecular oxygen with the formation of a phenol anthraquinone⁷ but, like vitamin B, it does not seem to be reducible.⁸ Vitamin C is not precipitated by lead acetate but is thrown down by sugar of lead [lead arsenate] in alkaline solution.⁹

Vitamin D is found in plants in active form only in insignificant amounts but it is a derivative of ergosterine and can easily be obtained from the latter in any quantity by the action of ultra-violet light.¹⁰ Ergosterine is present in large quantities in yeast. Avitaminosis D is the cause of rickets; with a deficiency of vitamin D, calcium is assimilated in insufficient amounts by the organism.¹¹

Vitamin D contains no nitrogen. After the irradiation of ergosterine with ultra-violet light, it is obtained in a yield of 10% at the most, which is explained as a destruction of the vitamin by the continued irradiation. Vitamin D is actually an unsaponifiable lipid but it is sharply distinguished from its parent substance—ergosterine—by its properties. It is less liable to oxidation than vitamins A and B.

Vitamin E, unlike the other vitamins, seems to produce no general disturbances when absent and merely exerts a powerful action on the functions

¹ Sherman and Burton. Jour. Biol. Chem. 70: 639. 1926.

² Coward. Jour. Biol. Chem. 72: 781. 1927; Moore. Biochem. Jour. 21: 870. 1927; Heller. *loc. cit.*; Crist and Dye. *loc. cit.*

³ Willimot and Wokes. Biochem. Jour. 20: 1299. 1926; Willimot. *Ibid.* 22: 67. 1928.

⁴ Lopez-Lomba and Randoin. Compt. rend. 175: 1003. 1923; Randoin. Bull. Soc. Hyg. Alim. 11: 463. 1923; Bezssonoff. Bull. Chim. Biol. 9: 555. 1927; Compt. rend. 186: 259. 1928; Scotti-Foglieni. Boll. Soc. Biol. Sper. Vol. 5. 1926; Archivio Fisiol. 26: 1. 1928.

⁵ Bezssonoff. Compt. rend. 180: 970. 1925.

⁶ Connel and Zilva. Biochem. Jour. 18: 644. 1924; Bezssonoff. *loc. cit.*

⁷ Bezssonoff. *loc. cit.*; Zilva. Biochem. Jour. 21: 689. 1927; 22: 779. 1928.

⁸ Bezssonoff. Compt. rend. 175: 846. 1922; Bull. Soc. Hyg. Alim. 11: 14. 1923; *loc. cit.*; 186: 259. 1928.

⁹ Bezssonoff. Compt. rend. 175: 846. 1922; 180: 970. 1925; Zilva. Biochem. Jour. 19: 589. 1925; 21: 354. 1927.

¹⁰ Knudsen and Moore. Jour. Biol. Chem. 81: 49. 1929; see also Windaus and Hess. Nachr. Ges. Wiss. Göttingen. 1: 175. 1927; Rosenheim and Webster. Biochem. Jour. 21: 389. 1927; Hess and Weinstock. Jour. Biol. Chem. 72: 301. 1924; 73: 297. 1925; Chick and Roscoe. Biochem. Jour. 20: 137. 1926; Roscoe. *Ibid.* 21: 211. 1927.

¹¹ McCallum, Simmonds, Becker and Shipley. Jour. Biol. Chem. 53: 293. 1922; Hess, Weinstock and Tolstoi. *Ibid.* 75: 731. 1923; Sherman and McLeod. *Ibid.* 64: 429. 1925; Chick, Korenchevsky and Roscoe. Biochem. Jour. 20: 622. 1926; Chick and Roscoe. *Ibid.* 20: 632. 1926; Karelitz and Shohl. Jour. Biol. Chem. 73: 655 and 665. 1927; Gyorgy. Klin. Wochenschr. Vol. 13. 1927.

of the organs of reproduction. It is therefore questionable whether this substance merits the name "vitamin." It is a lipoid-like, oxidizable substance which is soluble in the same solvents as vitamins A and D.¹ It seems that vitamin E consists of two components. When the first is lacking there is a degeneration of the male sperm-forming tissues and of the female egg cells; the other component is necessary for the normal activity of the mammary glands.²

It can scarcely be doubted that vitamins are a sharply defined group of physiologically important substances. It is obvious that vitamins have nothing to do with enzymes, *i.e.*, catalysts of single chemical reactions. The action of vitamins is extremely complicated and concerns various transformations of materials and energy. They also are certainly not identical with hormones which are to be considered in another volume. Hormones act as specific signals and so do not take part in characteristic physiological transformations of materials. On the contrary, the inability of animals to replace vitamins by other substances undoubtedly indicates that vitamins take a direct part in the biochemical transformations and do not merely serve as "chemical messengers."

It has already been pointed out that vitamins are also essential for plants. Since plants cannot be subjected to an artificial avitaminosis, the foregoing assumption is based only on indirect observations. For example, it has been reported that the growth of *Lemna minor* is extraordinarily accelerated after the addition of peat worked over by bacteria and supposedly rich in bacterial vitamins.³ Also the growth of wheat seedlings, the fermentation of yeast and the nitrogen fixation by *Azotobacter chroococcum* are said to be considerably stimulated by extracts of peat.⁴ It remains to be shown that it is vitamins which are active in these cases.

In aqueous extracts of yeast and in extracts of a few other plants, according to the statements of Wildiers,⁵ there is a substance of unknown nature which strongly stimulates the growth of yeast and is given the name *bios*. According to more recent data,⁶ this growth-promoting effect can be explained without assuming a specific substance.

¹ Sure. Jour. Biol. Chem. 74: 37. 1927.

² Evans and Bishop. Science. 56: 650. 1922; Jour. Metab. Res. 3: 281. 1923; Jour. Amer. Med. Assoc. 81: 889. 1923; Evans and Burr. Anat. Record. 27: 293. 1924; Proc. Nat. Acad. Sci. 40: 334. 1925; Sure. Jour. Biol. Chem. 58: 681. 1923; 62: 371. 1924; 74: 71. 1927.

³ Bottomley. Proc. Roy. Soc. London (B) 89: 481. 1917; Mockeridge. Biochem. Jour. 14: 432. 1920.

⁴ Bottomley. Proc. Roy. Soc. London (B) 88: 237. 1914; Kurono. Bull. Coll. Agric. Tokyo. 5: 305. 1915; Goy. Compt. rend. 172: 242. 1921; Abderhalden und Schaumann. Fermentforsch. 2: 120. 1918; 3: 44. 1919; Euler, v. und Karlson. Biochem. Zeitschr. 130: 550. 1922; [Breazeale, J. E. Univ. of Ariz. Agr. Exp. Sta. Tech. Bull. No. 16. 1927].

⁵ Wildiers, E. La cellule. 18: 311. 1901; Amand, A. Ibid. 21: 329. 1904; Devloo, R. Ibid. 23: 361. 1906; Kossowicz, A. Zeitschr. f. Landwirt. Versuchswesen Oesterreichs. 9: 668. 1906.

⁶ Medwedev, G. A still unpublished paper. [A critical review of the question of bios has been made recently by Wallace and Tanner. Centralbl. f. Bakt. (II) 76: 1-17. 1928-29. Their conclusion seems to be that the term "bios" is unnecessary but that the presence of organic material does help growth. A more precise piece of work is reported by Hammett, F. S. Protoplasma. 7: 297-322. 1929 from experiments with *Paramoecium*. In this case it was a question of growth by increase in cell number and it was reported that "sulfhydryl is the essential stimulus to growth." This substance was extracted, precipitated and also tested by synthetic compounds.—Ed.]

15. THE CHEMISTRY OF IMMUNITY

The investigation of immunity is a great branch of experimental science which cannot be fully explained here. Still some of the fundamentals of the chemistry of immunity may also be important for investigations in the field of plant physiology.

Whenever bacteria or their toxic metabolic products penetrate the body of an organism, they bring about the formation of specific "antibodies." The latter are formed not only in the case of a bacterial infection but also as a rule whenever various foreign animal or vegetable proteins are introduced. The "antibodies" must remove the foreign proteins;¹ this is the chemical nature of immunity. There are not many known cases in which plants are entirely immune.² The information at hand leaves no doubt that there is no essential difference between the immunity of animals and that of plants. As yet an immunity has been observed only against the proteins and bacterial protein precipitation. All proteins which induce the formation of antibodies are called antigens; among these, the so-called toxins³ deserve special attention. Toxins are strongly adsorbable colloidal proteins which are considerably more poisonous than all the inorganic or organic non-protein poisons. For example, guinea pigs are killed by 0.00000001 parts by weight of tetanus toxin, 0.000003 parts of diphtheria toxin or 0.00003 parts of plague or cholera toxin.⁴ The toxicity of toxins depends to a large extent on the conditions of growth of the particular bacteria and the presence of various substances in the substrate.⁵ Many bacteria also produce the so-called hemolysins which dissolve the blood corpuscles of animals. Among the seed-plants, *Euphorbiaceae* and *Leguminosae* have been thoroughly investigated for the presence of toxins. It was found that the toxins of seed-plants are also protein substances. The plant toxins often produce an agglutination of blood corpuscles;⁶ on the other hand, the agglutinins (p. 11) produced by animals often effect an agglutination of the entire cells of bacteria. N. Bernard⁷ believes that in the case of the digestion of the root fungi by seed-plants (p. 197 ff.) there is first an agglutination of the fungus cells. It should be noted that the flocculation of introduced foreign proteins is a general physiological reaction. It is produced by the so-called precipitins.⁸ These thermo-labile substances are produced only by the introduction of foreign proteins. Hence the precipitin reaction can be used as an aid in the classification

¹ This theory assumes that each organism has its specific proteins. It has already been pointed out (p. 284) that such an hypothesis is not improbable.

² On this point see Picato, C. Ann. Inst. Pasteur. 35: 893. 1921; [However, later work by Carbone, D. (Centralbl. f. Bakt. (II) 76: 428-437. 1928-29) and his students with 18 pairs of diseases and various host plants, such as *Begonia*, potatoes, oats, beans and wheat, showed that immunity which was sometimes complete could be produced by vaccination].

³ Roux et Jersin. Ann. Inst. Pasteur. 2: 629. 1888; 3: 273. 1889; and others.

⁴ Kruse. Allgemeine Mikrobiologie, p. 860. 1910.

⁵ Pasteur, L., Chamberland et Roux. Compt. rend. 92: 429. 1881; and many others.

⁶ Michaelis, L. und Steindorff. Biochem. Zeitschr. 2: 43. 1906; and others.

⁷ Bernard, N. Ann. Inst. Pasteur. 7: 369. 1909.

⁸ Bordet. Ann. Inst. Pasteur. 13: 240. 1899.

of plants. The reaction fails to work for closely related species and increases as the genetical relationship diminishes.¹

The foregoing processes are entirely unlike the reactions of enzymes. In the first place, the chemical processes taking place in immunity are produced directly by the specific substances which take part, while enzymes only change the rates of reaction. Secondly, the substances active in immunity are undoubtedly used up and are therefore not catalysts. At present it has not yet been made clear whether purely chemical or physical processes are the more important in immunity.

P. Ehrlich² claims that purely chemical processes are the basis of immunity. According to his conception, the toxins have a "toxophore group" which exerts a specific toxic effect on the protoplasm and, in addition, a "haptophore group" which has the capacity to unite with the side-chains of the protoplasmic proteins. As long as this union cannot take place, the protoplasm is not poisoned.³ The side chains of the protein which unite with the toxins are termed receptors by Ehrlich. As the result of a stimulation of the protoplasm by the fixing of the receptors, these side chains are said to be formed in excess; later they split off from the protein molecule and in this form can fix a considerable amount of toxins, thus decreasing the poisoning of the protoplasm. According to Ehrlich, the blood of immunized animals contains a large quantity of free receptors.

Other investigators believe that in the case of poisoning by toxins,⁴ adsorption phenomena play the leading rôle. It should be noted that the protoplasm can be poisoned not only by toxins but also by living bacteria. In all the cases there are at least two factors at work.⁵ The one is called the amboceptor and exerts a specific effect in so far as it catches certain bacteria which are later dissolved by the action of the second factor. This second factor is called the complement and is held as a thermo-labile colloidal substance in every blood cell. The complement can destroy bacteria only after the action of the amboceptor. However, the amboceptor appears in the blood only after infection with certain bacteria. Hence, there is here a complete analogy with the effect of receptors on toxins, yet a purely chemical explanation is hardly allowable in the present case because the action extends to whole bacterial bodies.

It may be assumed that the amboceptor is one of the easily adsorbed substances of the blood cells,⁶ or that a union of the amboceptor with the bacterial cells gives a substance with the properties of a hydrophobe colloid. As for the chemical nature of the complement, it would be very interesting to study this more closely, since complement is contained in the blood of every healthy animal. Arrhenius⁷ points out that the reaction between toxin and antitoxin should be investigated from a new standpoint because it is entirely similar to the process of neutralization of a base by a weak acid. In both cases an easily hydrolyzed product is said to be formed.

For other hypothetical substances which are said to be active in immunization, consult the special literature.

¹ Magnus, W. und Friedenthal. Ber. bot. Ges. 24: 601. 1906; 25: 242 and 337. 1907; 26a: 532. 1908; Idem. Landwirtschaftl. Jahrb. 38(V): 207. 1909; Relander, L. Kr. Centralbl. f. Bakt. (II) 20: 518. 1908; Wells, H. G. and T. B. Osborne. Jour. Infect. Diseases. 8: 66. 1911; Galli-Valerio und Bornand. Zeitschr. f. Immunitätsforsch. und exp. Therapie (I) 15: 229. 1912.

² Ehrlich, P. Ges. Arb. z. Immunitätsforsch. 1904.

³ According to some theories, the entire protoplasm of the cell is a giant molecule (cf. p. 313).

⁴ Field, C. W. and O. Teague. Jour. Exp. Med. 9: 86. 1907; Jacqué, L. et Zunz. Arch. Internat. de Physiol. 8: 227. 1909; Zunz. Bull. Acad. Roy. de Belgique. 1910; and others.

⁵ Bordet, J. Ann. Inst. Pasteur. 10: 193. 1895; 14: 257. 1900; 15: 289. 1901.

⁶ Arrhenius, S. Immunochemie. 1907; Idem. Ergeb. d. Physiol. 7: 480. 1908; Arrhenius und Madsen. Zeitschr. f. physikal. Chem. 44: 7. 1903.

⁷ Arrhenius, S. loc. cit.

In concluding the present consideration of substances in plants, the great importance of capillary and colloidal chemistry for advances in chemical plant physiology should be emphasized. The time is near when these branches of science will form the basis of all investigations of material transformations in living cells. In addition, the chemistry of preparation must solve the problems of the purification of colloids from their impurities, since the proof of the chemical nature of enzymes, vitamins, toxins and similar substances will soon become essential for any new and important investigations in the field of biochemistry.

CHAPTER VIII

RESPIRATION AND FERMENTATION

I. THE GENERAL CONCEPT OF PLANT RESPIRATION

The foregoing descriptions have dealt with various material transformations which involve the consumption of energy. In the physical physiology^m there will be explanations of the processes of plant growth, of coördinated differentiation of tissues and of various kinds of release of energy. These vital phenomena also require a constant supply of energy.

Of all the endothermal chemical reactions which take place in living plant cells, only the photosynthetic building of organic substances in the chloroplasts takes place directly at the expense of solar energy; otherwise the sources of energy are always certain specific chemical reactions which involve the liberation of heat. Of all these processes, the so-called oxygen respiration or normal respiration of plants is the most common.

By its nature, oxygen respiration is the antagonistic process to the photosynthetic building of carbohydrates. The solar energy stored during photosynthesis is set free by respiration. This expenditure of energy proceeds gradually but continually; all the vital functions are thereby set in motion. Plants without chlorophyll are also nourished with substances which were produced by green plants in the light; therefore in this case also, the solar energy caught by the chloroplasts is released in the process of respiration.

Lavoisier has already shown that the respiration of animals is only a slow combustion in which oxygen is consumed and carbon dioxide is formed. The classic investigations of Th. de Saussure¹ showed that plant respiration is essentially identical with that of animals, since there is the same gas exchange in plant respiration as in animal respiration.

Since respiration continues without interruption during the life of the plant, it involves a considerable consumption of the organic working materials. Full grown green plants make up this loss by the process of photosynthesis, while the chlorophyll-free lower plants replace this material by means of nutrients absorbed from their environment. However, in germinating seeds which respire at the expense of reserve substances, a loss of the respiratory material is directly noticeable. The dry weight of still non-assimilating seedlings is always considerably less than that of the seeds. This is evident from the following table taken from Boussingault:²

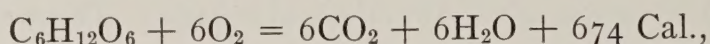
^m This is a reference to the second volume of this work as it appeared in the German edition.—*Ed.*

¹ Saussure, Th. de. *Ann. de Chim.* 24: 135 and 227. 1797; *Idem.* *Recherches chimiques sur la végétation*, p. 8 and 60. 1804.

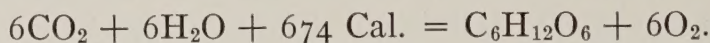
² Boussingault, J. B. *Agronomie, chimie agric. et physiol.* 4: 245. 1868.

Plant material	Dry wt. of seeds g.	Dry wt. of seedlings g.	Loss of weight during germination	
			g.	%
46 wheat seeds.....	1.665	0.712	0.953	57
1 kernel of corn "géant".....	0.5292	0.290	0.239	45
10 pea seeds.....	2.237	1.076	1.161	52

The analyses carried out by Boussingault proved beyond question that the loss of weight applies only to the carbon, hydrogen and oxygen. It is now known that sugars are the normal respiratory material and the entire respiratory process can therefore be expressed by the following equation:



while photosynthesis may be expressed by the following summary equation:



It is thus evident that, in the case of a total oxidation of sugar, oxygen respiration, in agreement with Lavoisier's conception, is to be regarded as a slow combustion. In the case of this analogy it must be borne in mind that the combustion of sugar in living plant cells is not like a funeral pile burning in the middle of a field but is more like a combustion of fuel in a steam engine. Only in the latter case is the heat of combustion not completely dispersed but by means of ingeniously designed apparatus, transformed partly into mechanical energy. Vital combustion in a plant cell also effects various transformations of energy and causes the mysterious apparatus of the living protoplasm to function. This is why we consider respiration as the criterion of life.

2. THE GAS EXCHANGE AND FORMATION OF WATER DURING RESPIRATION

Although the respiratory energy amounts to but a small fraction of the amount of photosynthetic energy of the green plants,¹ it is a matter of importance that the respiratory gas exchange continues day and night. On this account the consumption of respiratory material, calculated per unit of living substance, is very important. The respiratory energy is largely dependent upon the stage of development and the amount of living substance. In plant organs which are growing rapidly and which are rich in protoplasm, the respiratory metabolism is scarcely less than that of animals.

The amount of CO_2 given off from a unit of living substance per unit of time serves as a measure of the respiratory energy. Ordinarily one is content with a calculation of the CO_2 -production per gram of dry weight. Still it is evident that no great accuracy is possible with this kind of calculation. The percentage of protoplasm is not uniform in the various plants and parts of plants, the respiratory gas exchange of which depends entirely upon the activity

¹ Cf. e.g., Willstätter, R. und A. Stoll. Untersuchungen über Assimilation der Kohlensäure. 1918; Kostytschew, S. Ber. bot. Ges. 39: 319. 1921.

of the living substance. Only the cells of embryonic tissues are filled with protoplasm while in the cells of differentiated tissues the plasma at the most forms merely an inner lining of the wall. There are some tissues such as wood and cork, consisting largely of dead cells, which do not respire at all. On this account some investigators have attempted to develop more exact methods for the determination of the rate of respiration.

Palladin¹ attempted to explain the dependence of respiration on the amount of living substance through the determination, for the experimental material, of the amount of N in the proteins which are undigested by gastric juice. He regarded this nitrogen as the nitrogen of the nucleins which form the principal part of the protoplasmic structure. The value of $\frac{\text{CO}_2}{\text{N}}$, i.e., the amount of CO₂ given off from the experimental material divided by the nitrogen of the undigested protein, is, according to Palladin, the correct measure of the rate of respiration, i.e., of the CO₂ produced by the unit weight of protoplasm.

However, this kind of calculation also involves unavoidable sources of error. Gastric juice leaves undigested not only nucleins but also some reserve proteins such as prolamines. But in every case Palladin's method, in spite of objections on the part of various investigators, is more exact than the simple calculation of the carbon dioxide per unit of fresh or dry weight of the experimental material.

A still more accurate calculation might be possible, to be sure. For this purpose one should refer the respiratory carbon dioxide to the phosphorus of nucleins or to the purine nitrogen; but such determinations would never be carried out. They are so technically bothersome that they do not well merit consideration for numerous quantitative experiments. On the other hand, the simple calculation by total weight is quite permissible in cases in which one is dealing with materials which contain no great number of dead cells and exhibit no very great cellulose content. Useful comparative material yields such results in the case of investigations of lower organisms or the embryonic organs of seed-plants. The rate of respiration of various plants and plant parts is illustrated by the table on page 376, compiled from the experimental results of several investigators. The quantity of carbon dioxide liberated or of the oxygen absorbed is computed throughout per gram of dry weight.

From this table it is evident that in some cases the rate of plant respiration is not less than that of animal respiration. The respiration of microorganisms is particularly intensive during the period of rapid development. The mold *Aspergillus niger* is noted for its fabulous speed of growth. In 2 to 3 days rank, matted layers of mold develop on suitable nutrient solutions. The rate of respiration of *Aspergillus niger* is correspondingly extraordinary among the lower organisms.

In the case of seed-plants, the rate of respiration is also dependent to a marked degree upon the rate of growth. To be sure, resting seeds show a

¹ Palladin, W. Rev. gén. bot. 8: 225. 1896; 11: 81. 1899; 13: 18. 1901; Hettlinger, A. Ibid. 13: 248. 1901; Burlakoff. Arb. d. Naturforsch. Ges. in Charkow. Vol. 31. 1897 (Russian); and others.

decline in weight which is traceable in part to loss of water and in part to respiration, yet it is evident from the direct quantitative determinations of Kolkwitz¹ that the respiration of resting seeds is utterly unimportant. The rate of respiration of 1 kg. of seeds is 1 cc. of CO₂ in 24 hours with a water content of 10–11%. It is worthy of note that an increase of the water content of seeds to 33% increases the CO₂-formation to 1200 cc. per kilogram. Of course, it remains undecided whether this process is to be regarded as true respiration or merely as a spontaneous autoxidation of various labile substances contained in the seed coats.² Completely soaked seeds liberate very considerable amounts of CO₂.

Plant Material	Temp.	Rate of respiration in 24 hours
Very young wheat roots ¹	15–18°	67.9 cc. O ₂ absorbed
Older wheat roots ¹	15–18°	82.8 cc. O ₂ absorbed
Very young rice roots ¹	14–17°	44.4 cc. O ₂ absorbed
Older rice roots ¹	14–17°	55.1 cc. O ₂ absorbed
Roots of <i>Lamium album</i> ¹	18–19°	62.5 cc. O ₂ absorbed
Roots of <i>Mentha aquatica</i> ¹	18–19°	37.2 cc. O ₂ absorbed
Roots of <i>Caltha palustris</i> ¹	18–19°	19.1 cc. O ₂ absorbed
Leaves of <i>Phleum pratense</i> ¹	20–21°	27.2 cc. O ₂ absorbed
Leaves of <i>Lolium italicum</i> ¹	19–20°	24.8 cc. O ₂ absorbed
Leaves of <i>Phragmites communis</i> ¹	19–20°	12.8 cc. O ₂ absorbed
Leaves of <i>Veronica Beccabunga</i>	16–17°	24.8 cc. O ₂ absorbed
Leaf buds of <i>Syringa vulgaris</i> ²	15°	35 cc. CO ₂ liberated
Leaf buds of <i>Ribes nigrum</i> ²	15°	48 cc. CO ₂ liberated
Leaf buds of <i>Tilia curopaea</i> ²		66 cc. CO ₂ liberated
<i>Sphagnum cuspidatum</i> (moss) ³		32.9 cc. CO ₂ liberated
<i>Hyphnum cupressiforme</i> (moss) ³		17.2 cc. CO ₂ liberated
Germ. seeds of <i>Sinapis nigra</i> ²	16°	58.0 cc. CO ₂ liberated
Germ. seeds of <i>Lactuca sativa</i> ²	16°	82.5 cc. CO ₂ liberated
Germ. seed of <i>Papaver somniferum</i>	16°	122.0 cc. CO ₂ liberated
<i>Azotobacter chroococcum</i> ⁴		709.5 cc. CO ₂ liberated
<i>Aspergillus niger</i> 4 day cultures on quinic acid ⁵		276.1 cc. CO ₂ liberated
<i>Aspergillus niger</i> 3 day cultures on quinic acid ⁵		682.0 cc. CO ₂ liberated
<i>Aspergillus niger</i> 2 day cultures on quinic acid ⁵		1751 cc. CO ₂ liberated
<i>Aspergillus niger</i> 2 day cultures on quinic acid ⁵		1800 cc. CO ₂ liberated
<i>Aspergillus niger</i> 2 day cultures on quinic acid ⁵		1874 cc. CO ₂ liberated
<i>Bacillus mesentericus vulgatus</i> ⁶		1164.3 cc. O ₂ absorbed

¹ Freyberg. Landwirt. Versuchs-Stationen. 23: 463. 1879.

² Garreau. Ann. Sci. Nat. (3) 15: 1. 1851.

³ Jönsson, B. Compt. rend. 119: 440. 1894.

⁴ Stoklasa, J. Ber. bot. Ges. 24: 22. 1906; Centralbl. f. Bakt. (II) 21: 484. 1908.

⁵ Kostytschew, S. Jahrb. wiss. Bot. 40: 563. 1904.

⁶ Vignal, M. Contribution a l'étude des Bactériacées. 1889.

¹ Kolkwitz, R. Ber. bot. Ges. 19: 285. 1901; see also Muntz. Compt. rend. 92: 97 and 137. 1881.

² Becquerel, P. Compt. rend. 138: 1347. 1904; 143: 974 and 1177. 1906; Ann. Sci. Nat. Bot. (9) 5: 193. 1907.

In the course of the germination of the seed, the rate of growth increases during the first days, then gradually becomes smaller. The graphic representation of the process gives the so-called sigmoid curve of the grand period of growth. If one follows the course of respiration during the grand period of growth, one obtains the so-called grand curve of respiration of germinating seeds.¹ It is similar throughout to that of the grand curve of growth, although of course the liberation of CO₂ does not keep pace with the absorption of oxygen.² During the early days of germination the rate of respiration gradually increases, finally reaches a maximum and then decreases again toward the end of the germination. This connection between the curves is easily understood. The mechanical energy required for the formative processes of growth is developed in the respiratory processes. An increase of energy consumption requires a corresponding increase of energy production. Careful experiments showed that embryonic, growing organs respire more vigorously than endosperm.³ The rate of respiration decreases with advancing age of the plant.⁴

During the ripening of seeds the intensity of their respiration gradually becomes weaker and reaches a minimum value at the time of the complete dessication of the mature seeds.⁵

There is an intensive respiration in flowers⁶ which in general develop quickly and last but a short time. According to the data of de Saussure,⁷ sexual organs respire more vigorously than involucreal leaves. After pollination the respiration of the fruiting growth becomes very vigorous, a condition which is obviously connected with the formative processes in the organization of the embryo.⁸

The analyses of de Saussure show that the respiratory energy of flowers reaches a value of 2-4½ times that of foliage leaves.⁹ According to the most recent data, leaves which are rich in anthocyan exhibit especially vigorous respiration. With these the oxygen absorption is said to be very great.¹⁰ The chlorophyll-free, parasitic phanerogams respire fairly intensively according to the older researches of various workers. The rate of respiration of etiolated leaves fed with sugar is also quite high.¹¹

The ratio of the carbon dioxide thrown off to the oxygen taken in is called the respiratory quotient and is written $\frac{\text{CO}_2}{\text{O}_2}$. The value of this quotient is subject to various fluctuations which are not due to the antagonistic process of the photo-

¹ Mayer, A. Landwirt. Versuchs-Stationen. 18: 245. 1875; Rischawi. *Ibid.* 19: 321. 1876. The first determinations of the respiration of germinating seeds we owe to de Saussure. Mém. soc. phys. de Genève. 6: 557. 1833.

² Trietinger, G. Flora. 22: 167. 1927.

³ Burlakoff. Arb. d. Naturforsch.-Ges. Charkow. Suppl. 1 to vol. 31. 1897 (Russian).

⁴ Kidd, West and Briggs. Proc. Roy. Soc. London. 92: 368. 1921; Hover and Gustafson. J. Gen. Physiol. 10: 33. 1926-27.

⁵ Appleman and Arthur. Amer. Jour. Bot. 5: 207. 1918; Jour. Agric. Res. Vol. 17. No. 4. 1919.

⁶ de Saussure. Rech. chim. sur la végétation. 1804; Ann. de chim. et de physique (2) 21: 279. 1822.

⁷ de Saussure. *loc. cit.*; Cahours. Compt. rend. 51: 496. 1864; Maige, A. *Ibid.* 142: 104. 1906; Rev. gén. bot. 19: 9. 1907; Maige, G. *Ibid.* 21: 32. 1909.

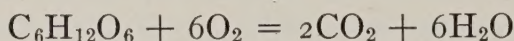
⁸ White, J. Ann. Bot. 21: 487. 1907.

⁹ de Saussure. *loc. cit.*

¹⁰ Nicolas, G. Compt. rend. 165: 130. 1918.

¹¹ Palladin, W. Rev. gén. bot. 5: 49. 1893; 6: 201. 1894.

synthetic assimilation of CO_2 in green leaves. The equation of oxygen respiration



states, on the basis of the law of Avogadro, that the volumes of CO_2 and O_2 in the gas exchange should be equal. This holds in many cases, but various deviations from the regularity are possible. This can come from the following reasons.

I. Simultaneously with the oxygen respiration there also takes place an independent absorption of oxygen for other purposes. The extra oxygen is used in the formation of organic carboxylic acids and other compounds rich in oxygen. In all such cases $\frac{\text{CO}_2}{\text{O}_2}$ is less than 1. As a common example of this type, there are those respiratory processes which occur in seed germination,¹ just as in general during vigorous growth and vegetative development.² The storage of organic acids in ripening fleshy fruits and in succulents³ also effects a depression of the value of $\frac{\text{CO}_2}{\text{O}_2}$.

II. In other cases, on the contrary, a surplus of CO_2 is formed by processes which proceed without an absorption of oxygen. Very commonly aerobic respiration and alcoholic fermentation take place simultaneously. With this condition $\frac{\text{CO}_2}{\text{O}_2}$ is greater than 1. During the early stages of germination of some seeds whose compact seed-coat is scarcely permeable to oxygen, alcoholic fermentation stands out as the normal process of germination, so long as the seed-coat is not pierced by the radicle.⁴ Analogous phenomena are to be found in the respiration of yeast⁵ and some of the *Mucoraceae*.⁶ Here there is oxygen respiration as well as alcoholic fermentation and $\frac{\text{CO}_2}{\text{O}_2}$ is greater than 1.

III. Various fluctuations in the value of $\frac{\text{CO}_2}{\text{O}_2}$ may originate as the result of the given respiratory material having a percentage composition different from that of sugar. This is the most common basis of "abnormal" size of $\frac{\text{CO}_2}{\text{O}_2}$. For example, after the complete consumption of the sugars and by way

¹ Bonnier, G. et Mangin. Ann. Sci. Nat. (6) 18: 364. 1886.

² Palladin, W. Ber. bot. Ges. 4: 322. 1886; Constamm. Rev. gén. bot. Vol. 25 as far as p. 539. 1914.

³ Aubert. Rev. gén. bot. 4: 378. 1892; Gerber. Compt. rend. 124: 1160. 1897; Ann. Sci. Nat. Bot. (8) 4: 1. 1896.

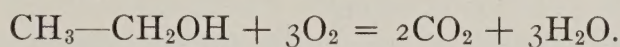
⁴ Polowzow, V. Untersuchungen über Pflanzenatmung. 1901; Kostytschew, S. Biochem. Zeitschr. 15: 164. 1908; Idem. Physiol.-chem. investigations in plant respiration. 1910 (Russian).

⁵ Buchner, E., H. Buchner und M. Hahn. Die Zymasegärung, p. 350. 1903; [Meyerhof, O. Biochem. Zeitschr. 168: 43. 1925].

⁶ Kostytschew, S. Centralbl. f. Bakt (II) 13: 490. 1904; Kostytschew und P. Eliasberg. Zeitschr. f. physiol. Chem. 111: 141. 1920.

ⁿ Another cause for fluctuations is the amount of water contained in the tissues, according to Mayer, A. et L. Plantefol. Compt. rend. 181: 131-132. 1925. In general the respiratory quotient varies inversely with the total amount of water imbibed.—Ed.

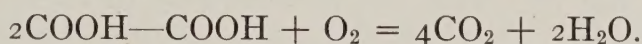
of a satisfactory substitute, the plants begin to burn the protein material of their protoplasmic framework, and in consequence $\frac{\text{CO}_2}{\text{O}_2}$ falls to 0.70–0.80: that is to say, protein is poorer in oxygen than sugar and so uses up, for complete combustion to CO_2 , H_2O and N , a greater quantity of atmospheric oxygen per unit weight. In other cases there is consumption of the ethyl alcohol which is split off under unfavorable conditions of aeration (see above). The total combustion of alcohol involves a lower value of $\frac{\text{CO}_2}{\text{O}_2}$:



$$\frac{\text{CO}_2}{\text{O}_2} = \frac{2}{3} = 0.66.$$

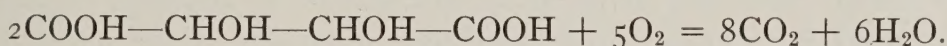
This process stands out as the normal phenomenon in the germination of pea seeds up to the time that the compact seed coats burst. The alcohol which is split off in the first stages of germination (see above) is burned with a considerable oxygen consumption.¹ In this case the respiratory quotient is very small.

In other cases, however, materials rich in oxygen, such as organic acids, may proceed to a complete combustion, which would bring about an appreciable increase in the value of $\frac{\text{CO}_2}{\text{O}_2}$. For example, the respiratory quotient for the complete oxidation of oxalic acid is theoretically 4:



$$\frac{\text{CO}_2}{\text{O}_2} = \frac{4}{1} = 4.$$

In the case of this oxidation the heat production is quite insignificant. In the oxidation of tartaric acid $\frac{\text{CO}_2}{\text{O}_2}$ equals 1.6:



$$\frac{\text{CO}_2}{\text{O}_2} = \frac{8}{5} = 1.6.$$

Further on the evidence will be brought out that all reserve, nitrogen-free substances consumed by respiratory processes are burned by way of the intermediate stage of sugar. By this means the theoretical value of the respiratory quotients of the above materials will naturally not be influenced. It is clear that in the case of the transformation of a non-carbohydrate into sugar exactly the same amount of oxygen is separated or consumed which, with a direct combustion, brings about the “abnormal” value of the respiratory quotient.

Hence, in the respiration of oily seeds, low values of $\frac{\text{CO}_2}{\text{O}_2}$ would be observed,²

¹ Kostytschew, S. *Physiol.-chem. investigations in plant respiration*. 1910 (Russian).

² Godlewski, E. *Jahrb. wiss. Bot.* 13: 491. 1882.

a condition which is connected with the fact that fats are changed to sugars.¹ If fatty seeds are fed with prepared sugar, $\frac{\text{CO}_2}{\text{O}_2}$ reaches the normal value of 1, since under these conditions a direct combustion of sugar ensues and the respiration of fat is inhibited.² In the case of the ripening of oily seeds, on the contrary, the quotient is greater than 1 because carbohydrate is being changed to fat and a certain amount of oxygen is set free. Accordingly the absorption of atmospheric oxygen for respiration must decrease, since a part of the carbon compounds are oxidized to CO_2 by the oxygen left over from the formation of fat.³

For experimental studies of $\frac{\text{CO}_2}{\text{O}_2}$, the rapidly growing and energetically respiring molds are very suitable. Experiments carried out with them have proved that the respiratory quotient is not exactly the same for various sources of carbon.⁴ With the very abundant respiration of *Aspergillus niger*, the expected theoretical value of $\frac{\text{CO}_2}{\text{O}_2}$ for various organic nutrients can be established by analyses.⁵

On the basis of this evidence we may conclude that the heat production of respiration may not always be due to a direct combustion of sugar. This is true only when $\frac{\text{CO}_2}{\text{O}_2}$ is equal to 1, while $\frac{\text{CO}_2}{\text{O}_2} > 1$ indicates a lower heating effect.

As to what a value less than 1 indicates, no definite conclusion is possible since the low value of the respiratory quotient may be due to various conditions. If a complete combustion of an oxygen-poor respiratory material (such as fat) takes place the heating effect is, of course, very great but if only an oxygen-tying action, which does not result in complete combustion, is involved, the heating effect in such cases remains small.

Unfortunately the formation of water in plant respiration has been insufficiently studied. This is all the more to be regretted since the amount of water formed in many cases can serve as a criterion of a complete combustion of respiratory material. Direct analytical determinations of the water formed in the respiratory processes are to be found in the older work of Liaskowski,⁶ yet the question of water formation in respiration is not settled by them since the hydrolytic processes taking place in seed germination evoke appreciable but unexplored fluctuations of water formation.⁷ Experimental studies of water formation are rendered difficult by reason of the complicated methods and manifold sources of error; in spite of this there is an urgent need to remedy this omission.

¹ Liaskowski, N. Chemical investigations of the germination of pumpkin seeds. 1874 (Russian).

² Polowzow, V. Investigations in plant respiration. 1901 (Russian).

³ Godlewski, E. *loc. cit.*

⁴ Diakonow, N. Ber. bot. Ges. 5: 115. 1887; Puriewitsch, K. Jahrb. wiss. Bot. 35: 573. 1900.

⁵ Kostytschew, S. Jahrb. wiss. Bot. 40: 563. 1904.

⁶ Liaskowski, N. *loc. cit.*; see also Landwirtschaftl. Versuchs-Stationen. 17: 219. 1874. [For a brief description of the experiments of Liaskowski see Palladin's Plant Physiology (transl. by Livingston) 2nd. ed., p. 217-218.]

⁷ A strong proof of the existence of such hydrolytic processes resulted from Bonnier's studies of heat production during seed germination (Bonnier, G. Ann. Sci. Nat. Bot. (7) 18: 1-35. 1893).—Ed.

3. PRODUCTION OF RADIANT ENERGY IN PLANT RESPIRATION

Plant respiration, like all slow combustions, involves a production of heat. The amount of heat depends upon the given respiratory material. Since in plant respiration it is usually carbohydrate which serves as the direct material for combustion, we may select as the point of departure for theoretical considerations the heat of combustion of 1 molecule of grape sugar. This amounts to 674 Calories.¹ It was pointed out above that we may assume a direct oxidation of sugar only when $\frac{\text{CO}_2}{\text{O}_2}$ is exactly 1 as shown by the gas analyses.

Plants have no thermo-regulatory contrivances. For that reason the temperature of the plant body is for the most part hardly different from that of the surrounding medium. Hence, at first sight, it appears that plant respiration produces no heat. In some cases in which moderately large plant organs evince a considerable respiratory process, a direct increase in temperature is at once perceptible.² Thus a direct measurement of temperature shows that in *Cereus* flowers between the filaments there is a temperature rise of 1.2°.³ In the flowers of *Victoria regia* a temperature increase of 12.5° has been observed⁴ and in the flower spadices of *Arum italicum* a rise of 36°. The temperature of the spadices then reaches 51° in an air temperature of 15°.⁵ The heat production of *Arum* can be further increased by wounding.⁶ Also in the blossoms of *Ceratozamia* and some palms there is a considerable release of heat.⁷ Indeed it is easy to demonstrate a considerable production of heat by many other kinds of plants if the temperature is measured in a Dewar flask or a similar method is used to prevent dispersion of the heat. In this way it is possible, for example, to obtain a temperature of 50° with foliage leaves.⁸ Similar temperatures have been observed for the respiration of seeds of various cereals. The heating of hay, which reaches a very high point, is evoked by various bacteria which produce an enormous amount of heat.⁹ In the case of some plants, respiration is accompanied by the formation of light as well as heat. The great British physicist Boyle has already noted that rotting wood does not glow in an air-free medium. The phosphorescence of plants is actually a result of oxygen respiration. Included among the phosphorescent plants are some fungi such as certain species of *Agaricus*, *Polyporus*, *Auricularia* and others,¹⁰ not

¹ Stohmann, F. Jour. f. prakt. Chem. 19: 115. 1879; 31: 273. 1885; Zeitschr. f. Biol. 13: 364. 1894.

² de Saussure. Ann. Sci. Nat. 21: 285. 1822; Ann. de chim. et de physique (2) 21: 279. 1822; Dutochet. Compt. rend. 8: 741. 1839; 9: 613. 1839; Ann. Sci. Nat. (2) 13: 1. 1840; and others.

³ Leick, E. Ber. bot. Ges. 33: 518. 1915; 34: 14. 1916.

⁴ Knoch, E. Untersuchungen über die Morphologie und Biologie der Blüte von *Victoria regia*, p. 38. 1897.

⁵ Kraus, G. Abh. d. Naturforsch.-Ges. Halle. Vol. 16. 1882; Ann. Jardin Bot. Buitenzorg. 13: 217. 1896.

⁶ Sanders, C. B. Report Brit. Assoc. York, p. 739. 1906.

⁷ Kraus, G. loc. cit.

⁸ Molisch, H. Bot. Zeit. 66: 211. 1908; Zeitschr. f. Bot. 6: 305. 1914.

⁹ Citations by Miehé, H. Die Selbsterhitzung des Heues. 1907; see also Gorini, C. Atti. d. Reale accad. dei Lincei, rendiconto (5) 23 (1): 984. 1914; Burri, R. Landwirtschaftl. Jahrb. 33: 23. 1919; and others.

¹⁰ Smith, W. G. Gard. Chronicle. 7: 83. 1877; Crié, L. Compt. rend. 93: 853. 1884; Kutscher, F. Zeitschr. f. physiol. Chem. 23: 109. 1897; Atkinson. Bot. Gaz. 14: 19. 1889; and others.

to mention some algae and specific bacteria (*Photobacterium* and others).¹ The phosphorescent bacteria soon perish on the usual nutrient media; the phosphorescence revives in cultures on fish bouillon. Not until recently was this phosphorescence observed on artificial substrates of known composition.² It takes place only within fixed temperature limits. It is worthy of note that phosphorescent bacteria often belong to the group of decay fungi which grow well on proteins alone (*i.e.*, in the absence of carbohydrates). The intensity of the bacterial light is sufficient to evoke the formation of chlorophyll in etiolated plants.³

The nature of the phosphorescence consists in this—that in the respiration of the phosphorescent plants there is a formation of certain materials which glow after death and are called luciferins. Their chemical nature is not yet established but it is very probable that they are to be classed in the group of protein compounds⁴ and are formed under the influence of specific enzymes.⁵ Moreover, it is well-known that a few organic compounds glow with slow oxidation.

Various workers have endeavored to determine quantitatively the respiratory energy used up in plants. It is clear that the fraction of the total energy of respiration which is not dispersed in the form of radiant energy but is used in the protoplasm for various vital needs, may, under some conditions, be moderately small. We know how insignificant is the economic quotient [efficiency] of our steam engines and other heat apparatus. The first calorimetric determinations were carried out with resting organs;⁶ no wonder that in this case the radiant energy produced by the respiration was practically equal to the total heat of combustion of sugar. The following table of Rodewald shows the quantity of heat which corresponds to the production of 1 cc. of CO₂ or the absorption of 1 cc. of oxygen.

CO ₂ given off	O ₂ absorbed	$\frac{\text{CO}_2}{\text{O}_2}$	Heat produced in Calories	Heat in Cal. per cc. CO ₂	Heat in Cal. per cc. O ₂
6.175	5.842	1.06	30.3	4.91	5.19
4.883	4.354	1.12	19.7	4.03	4.53
4.625	4.507	1.03	19.6	4.24	4.35

The determinations of Bonnier⁷ showed an even greater heat production as it corresponds to the total oxidation of sugar (respiration is, as we know, not the only source of energy of living cells). The recent thorough studies of

¹ Beijernick. Medd. Akad. Amsterdam. No. 2, p. 7. 1890; Molisch, H. Leuchtende Pflanzen. 2nd ed. 1912.

² Chodat, R. et de Coulon. Arch. Sci. Phys. et Nat. Genève (4) 41: 237. 1916.

³ Issatschenko, B. Abh. d. bot. Gartens in St. Petersburg. 11: 31 and 44. 1911 (Russian).

⁴ Dubois, R. Compt. rend. 111: 363. 1890; 123: 653. 1896; 153: 690. 1911; 165: 33. 1917; 166: 578. 1918; Compt. rend. soc. de biol. 81: 317. 1918; 82: 840. 1919.

⁵ Dubois, R. *loc. cit.*; Harvey, E. N. Amer. Jour. Physiol. 44: 449. 1916; 45: 318, 342 and 349. 1917; J. Gen. Physiol. 5: 275. 1923.

⁶ Rodewald. Jahrb. wiss. Bot. 18: 263. 1887; 19: 221. 1888; 20: 261. 1889.

⁷ Bonnier, G. Ann. Sci. Nat. (7) 18: 1. 1893.

L. C. Doyer¹ yielded still other results. It was shown that, during the periods in which intense growth and formative processes are taking place, as is the case for example during the early days of seed germination, the greater part of the respiratory energy is used for vital needs and only a little energy escapes in the form of heat: *e.g.*,

WHEAT SEEDLINGS

Day of germination	Respiratory energy per kg. at 25° (accord to CO ₂ -pro- duction) in Cal.	Energy directly measureable as heat per kg. at 25° in Calories
2	2135	363
3	3802	540
4	6277	2938
5	6886	3216
6	8837	4341

It is therefore evident that on the second day of germination only 12% of the total energy is released as heat. Most of the chemical energy is used in the formative processes. Even on the sixth day only half of the total energy could be measured as heat.

According to the latest determinations, molds liberate most of the respiratory energy as heat.² However, in this case old fungus mats were employed for the experiments. Probably young, vigorously growing fungus mats would yield entirely different results.

4. ANALYTICAL METHODS FOR THE DETERMINATION OF OXYGEN RESPIRATION

The qualitative determination of the liberation of CO₂ by plants offers little interest. Only quantitative determinations of plant respiration have real physiological significance since they make it possible to follow the fluctuations of the rate of respiration and of the value of $\frac{\text{CO}_2}{\text{O}_2}$ under different conditions.

The quantitative determinations either measure the carbon dioxide liberated per unit of time (determination of the rate of respiration) or give the value of $\frac{\text{CO}_2}{\text{O}_2}$ which requires the simultaneous determinations of CO₂ and O₂.

For determinations of the rate of respiration, the experimental material is placed in a suitable container with an entry and an exit tube and left for a time in a current of air free from CO₂. The carbon dioxide which is formed is caught in absorption apparatus. In order to remove all the CO₂ from the current of air, a drying tower filled with soda lime is introduced ahead of the plant container. Barcroft's method and its later modifications, which are used in animal physiology, are only useful in plant physiology for investigations in photosynthe-

¹ Doyer, L. C. *Madd. Akad. Wet. Amsterdam*. 17: 62. 1914; *Recueil des travaux bot. néerland.* 12: 372. 1915.

² Molliard, M. *Compt. rend. soc. de biol.* 87: 219. 1922.

sis. Only too frequently, various plants resorb considerable quantities of oxygen not for combustion of material but for other purposes. Hence the rate of plant respiration can be measured only on the basis of the output of CO_2 and not by the absorption of oxygen. The large variations in pH which take place in plants, especially in the case of microorganisms, may also introduce serious errors into manometric experiments. Such rapid changes in reaction are definitely prevented in the blood of animals and the Barcroft apparatus can therefore be used. Only chemical methods for determinations of CO_2 and O_2 are really reliable for investigations in plant respiration.

If a more common type of absorption vessel is not necessary, and the quantity of CO_2 developed is considerable, the Geissler potash or soda lime tubes may be used to advantage, weighing them before and after the experiment. With these methods of determining CO_2 , the gas must enter the absorption tubes in a completely dry condition.

On the contrary, if it is desired to study the course of respiration in detail by rapid, successive determinations of CO_2 and eventually to plot them, a constant interchange of the foregoing absorption apparatus, besides the numerous

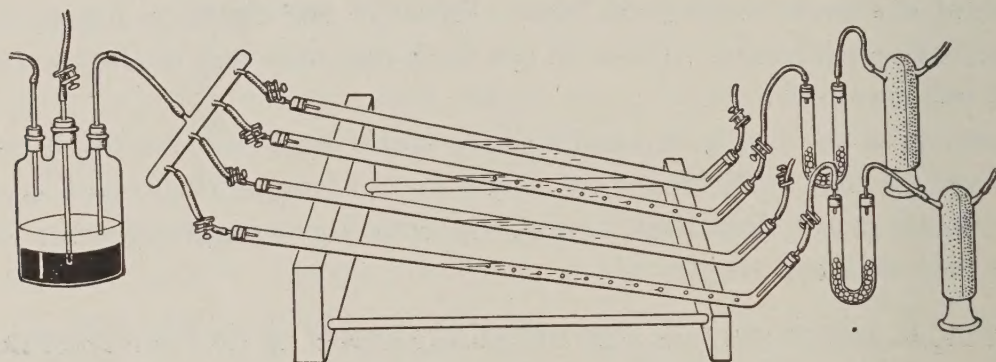


FIG. 37.—Pettenkofer tubes. Explanation in the text.

weighings, is found to consume considerable time. For such experiments one uses for the most part the so-called Pettenkofer tubes¹ which are made in various forms. The most useful model is shown in Fig. 37. Two tubes belong to each experimental chamber and these are prepared with titrated barium hydroxide^p and fastened in the inclined position as shown in the figure. The CO_2 -free air passes into the experimental chamber (in the figure a U-tube is used to hold the plants) and then into the Pettenkofer tube where the carbon dioxide is absorbed. The second tube in the meantime is left unused. After a certain time has elapsed, the first tube is cut out and the plant container is at once connected with another fresh Pettenkofer tube. The tube taken out is emptied by shaking into a dry glass bottle with a ground and greased glass stopper. After the precipitate has settled, the clear solution is titrated and the

¹ Pettenkofer, M. Abh. d. Bayer Akad. (II) 9: 231. 1862; Pfeffer, W. Untersuch. aus. d. bot. Inst. Tübingen. 1: 636. 1881-1885.

^p As an alternative to the use of barium hydroxide, Nishi (Jour. Biochem. Tokyo. 4: 473-480. 1925) describes the use of $\frac{N}{50}$ NaOH which is titrated in the presence of NaHCO_3 . Since thymolphthalein becomes blue at the pH of a dilute solution of NaHCO_3 , its use permits the titration of the NaOH to completion without a bicarbonate error.—Ed.

amount of absorbed carbon dioxide is thus measured. It is clear that one can work in this way for any length of time by constantly changing tubes, and the rate of respiration can be measured by frequent determinations. The plant container may be of any form and size according to the plant material used. The volume of the vessel must be such that CO_2 cannot accumulate in it with the ordinary rate of passage of a current of air (about 2 liters per hour).

For determinations of the value of $\frac{\text{CO}_2}{\text{O}_2}$, the experimental material is placed in vessels from which samples of gas can be removed. The CO_2 and O_2 content of the latter are determined by gas analyses. For accurate experiments the internal atmosphere must be separated from that without by means of glass and mercury. Small objects are inclosed in graduated analysis tubes in the same way as in experiments in photosynthesis. The flask shown in Fig. 38 is recommended for large amounts of material as follows; the distension C in the neck of the flask above the stopper is filled with mercury, the manometer tube *b* is inserted in mercury and the entrance tube *a* together with the attached rubber tubing *d* is filled with mercury, whereat the mercury in the manometer is then brought to a certain height by removing the corre-

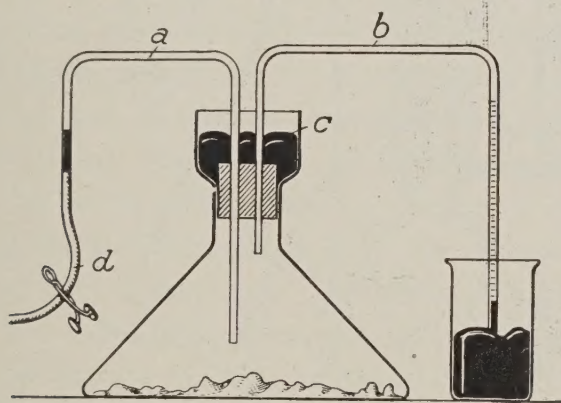


FIG. 38.—Kostychev's flask. Explanation in text.

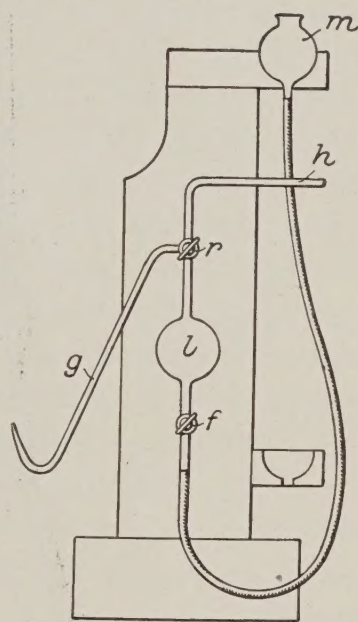


FIG. 39.—Kostychev's gas pipette. Explanation in text.

sponding sample of gas. The special gas pipette (Fig. 39) is used to remove the sample of gas from the plant container.

By the air of the three-way stopcock *r*, the bulb *l* can be connected with either the tube *g* or the tube *b*. The single stopcock *f* connects the bulb *l* with the pear-shaped bulb *m*. The pipette is screwed to a stable wooden frame.

With the aid of the pear-shaped bulb *m*, the bulb *l* and both tubes *g* and *h* are filled with mercury. For this purpose the pear-shaped bulb is elevated, the bulb *l* connected with the tube *g* and the stopcock *f* opened. As soon as the bulb and the whole tube are filled to the further aperture with mercury and a drop of mercury projects out, the stopcock *r* is so turned as to connect the tube *h* with the bulb and to fill this tube with mercury, after which the single stopcock *f* is closed. The curved end of the tube *g* is then dipped in a thick-walled vessel of mercury and the tube *h* is connected with the rubber tubing *d* (Fig. 38)

of the respiration flask just fitted out for the experiment. A certain amount of mercury is drawn through it by lowering the pear-shaped bulb *m* and opening the glass stopcock *f*. As a result the mercury in the manometer tube *b* of the flask rises to a certain level. The stopcock *f* is now closed, the bulb *l* is connected with the tube *g* by a suitable turn of the three-way stopcock *r*, the pear-shaped bulb *m* is elevated, the stopcock *f* is opened, and the last trace of gas is driven out of the bulb and the tube. After closing the stopcock *f*, the bulb *l* is again connected with the tube *h* and this tube and the attached vertical arm *a* of the flask tube are filled with mercury. The necessary manipulations are

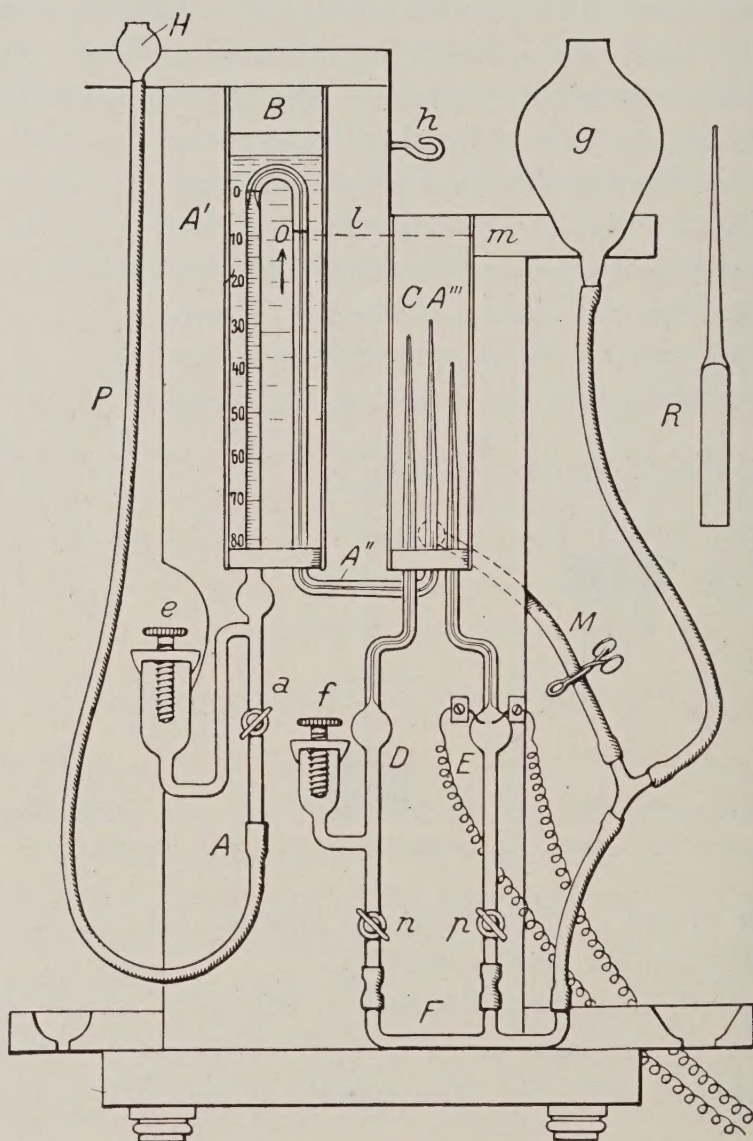


FIG. 40.—The Polowzow-Richter apparatus. Explanation in the text.

intelligible from the explanation above: the pear-shaped bulb *m* is raised and the stopcock *f* is opened. The experimental flask is now perfectly sealed as shown in the figure. The barometer reading and the level of mercury in the manometer tube are noted and the experiment begins therewith. This sealing of the flask is really very simple. After a little practice it is easily and safely performed in less time than when it is necessary to follow the above explanation.

The experiment proper consists in withdrawing, from time to time, test portions of gas from the experimental flask as described above, and in passing them through the tube *g* into the analysis tubes completely filled with mercury. This procedure is entirely analogous to the original closing the flask except that in the latter case the gas sample is not kept for analysis.

The sample of gas removed from the experimental chamber is analyzed in an apparatus suitable for the purpose. According to the experience of some, the Polowzow-Richter apparatus (Fig. 40) is recommended because it yields very accurate results, an analysis is possible with very small quantities of gas and it can be made by any experienced glass blower at a moderate price.¹ The gas to be analyzed is passed from the gas pipette (Fig. 39) into a small analysis tube with a fused-in handle (Fig. 40R). The analysis tube is carried over to the mercury bath *C* of the Polowzow-Richter apparatus, placed under mercury on the end *A'''* of the measuring tube *AA'A''A'''* and the gas drawn into the measuring tube by lowering the pear-shaped bulb *H* and opening the stopcock *a*; there the total volume of the gas sample is accurately measured by the aid of a kathetometer. The gas is then passed by way of the analysis tube into the absorption pipette filled with potassium hydroxide [or NaOH]; for this purpose the analysis tube is placed under mercury on the end of the pipette, the pear-shaped bulb *G* is lowered and the stopcock *n* is opened. After about 10 minutes the gas is driven from the pipette back into the measuring tube and again measured. The difference between the two readings gives the quantity of carbon dioxide absorbed by the potash. The remainder is added to an accurately (in the measuring tube) measured amount of pure hydrogen prepared by electrolysis, and the mixture is exploded in the explosion pipette *E*. A third of the loss in volume after the explosion is due to the oxygen contained in the gas to be analyzed. The screws *e* and *f* which are set in mercury make possible the delicate movements of the gas or mercury in the closed capillary tube. The screw *e* facilitates the adjustment at the zero point of the measuring tube while the screw *f* prevents a contamination of the analysis tube with potash during the displacement of gas from the pipette *D* into the analysis tube after the absorption of CO₂. The glass bulb *G* is connected by rubber tubing and a T-tube with both the mercury bath *C* and the two small pipettes *D* and *E*. It is therefore possible by means of this bulb to fill or to empty the bath *C* or to draw gas into the pipettes or to displace it.

The gas analyses yield the percentage content, but not the absolute amounts of the single components of the gas mixture which is being studied. These are needed, however, for the determination of the value of $\frac{\text{CO}_2}{\text{O}_2}$. This calculation is based on the fact that in respiration there is neither an absorption nor a liberation of nitrogen. We assume that *a* is the percentage of CO₂, *b* is the percentage of oxygen, and *c* is the percentage of nitrogen and other inert gases in the gas mixture just analyzed. Let the oxygen content of the atmosphere be O₂ and

¹ More exact directions for using the Polowzow-Richter apparatus can be found in **Kostychev's** *Pflanzenatmung*. 1924. [Plant Respiration (translated by C. J. Lyon). Blakiston. 1927.]

the nitrogen content N_2 . If the total volume of the gas mixture has changed during the experiment, the amount of oxygen absorbed equals $\frac{O_2 \cdot c}{N_2} - b$. If we express $\frac{O_2}{N_2}$ by q , the following equation results:

$$\frac{CO_2}{O_2} = \frac{a}{cq - b}.$$

In some experiments, however, one must determine the absolute quantities of oxygen absorbed and carbon dioxide liberated. For this purpose it is necessary to determine the total volume of the gas mixture in the container as well as the percentages of the single constituents. Since a direct calibration of the experimental chamber together with the experimental material contained therein is in most cases not feasible, the volume of the inclosed space is determined as follows. Before the experiment is started a gas sample v , measured at atmospheric pressure H in a calibrated measuring tube, is drawn from the flask. The lowering of the pressure in the flask which results from the removal of the test portion of gas is then noted with the aid of the manometer tube. Let h be the gas pressure in the flask before, and h' the pressure after the removal of the portion v . The desired total volume x of gas in the flask is calculated from these data on the basis of the law of Boyle-Mariotte:

$$x = \frac{vH}{h - h'}.$$

Then $xh = xh' + vH$.

If the volumes of the oxygen absorbed and the carbon dioxide liberated are known, the amounts by weight of both gases are obtained by simple calculations.

If very large amounts of plants are being used for investigation, or if it is impossible to introduce the research material into the neck of the flask without damaging it, the flask is replaced by a bell jar, the upper opening of which is closed with a two-hole rubber stopper. Into the holes of the stopper are introduced the same tubes as were referred to in the description of the respiration flask. The lower rim of the bell jar must be fitted to a ground glass plate by means of vacuum grease. For accurate experiments the bell jar together with the glass plate is placed in a crystallizing dish of mercury in order to obtain a perfect seal.

In using very small quantities of material, thick-walled test tubes can be used to advantage. The material is introduced into the upper part of the tube and held in position with glass wool. Then the procedure goes according to the general principles of gasometric methods.

5. THE INFLUENCE OF VARIOUS EXTERNAL FACTORS ON OXYGEN RESPIRATION

It has been pointed out above that respiration as a constant feature of life is a criterion of the living condition of cells. The modifications of the respiratory process under the influence of external factors might often serve as a direct measure of the reaction of the living protoplasm to various stimuli. Hence,

various kinds of studies of the action of external stimuli on respiration have been carried out by many investigators.

The influence of temperature on respiration. Many plants still respire at temperatures which are far below 0° .¹ Thus the respiration of the leaves of conifers and *Viscum* continues at -20° ,² although at a very slow rate. An increase in temperature always effects an increase in the rate of respiration in connection with which for the most part³ the law of van't Hoff³ prevails, that the temperature coefficient^r is 2-3 as for all reactions of organic materials. Contrary to the opinion of various workers, it can now be considered as proved that there is no optimum temperature for plant respiration. The amount of carbon dioxide given off increases regularly with the gradual increase in temperature, finally reaches a limiting value and remains at that level until the plant dies from the high temperature⁴ (at about 50°). Marked temperature fluctuations effect an increase in the rate of respiration.⁵ It can hardly be doubted that we are dealing here with a complicated irritation effect (*Reizwirkung*). The same explanation also holds for the influence of warm baths on respiration.⁶ The warm bath is a strong irritation effect which is used in horticultural practice to shorten the latent period of winter.

If a complete combustion of respiratory material takes place in respiration, $\frac{CO_2}{O_2}$ remains perfectly constant for different temperatures.⁷ It does not remain

¹ Kreusler, M. Landwirt. Jahrb. 17: 161. 1888.

² Maximow, N. Bot. Jour. d. Naturforschges. in Petersburg, p. 23. 1908 (Russian).

³ The agreement is closer for temperatures below $25^{\circ}C$.; cf. especially Kuyper. Rec. trav. bot. Néerland. 7: 130. 1910 which is a report of the most exact work done up to that time.—Ed.

⁴ Matthaei, G. L. C. Phil. Trans. Roy. Soc. (B) 197: 47. 1904; Blackman, F. F. and Matthaei. Proc. Roy. Soc. London (B) 76: 402. 1905; Smith, A. M. Proc. Camb. Phil. Soc. 14: 296. 1907; Crozier. J. Gen. Physiol. 7: 123. 1924-25.

⁵ The term "temperature characteristic" has been proposed by Crozier (*loc. cit.*) as a name for the quantity μ , or E , in the Arrhenius-Marcelin-J. Rice equation for velocity of a chemical reaction as controlled by temperature, this being of greater theoretical significance, and more constant in many cases, than the Q_{10} ratio. From the results of numerous experimental studies and analyses of existing records, Crozier has calculated μ (the critical thermal increment) of the Arrhenius equation

$$\frac{K_2}{K_1} = e^{\frac{\mu}{2} \left(\frac{1}{T_1} - \frac{1}{T_2} \right)}$$

for a number of vital processes including respiration. It appears that there are two, probably three, well-defined "temperature characteristics" for respiration, 11,500, 16,200 and 8,000 (Crozier. J. Gen. Physiol. 7: 189-216. 1924-25). Each of these may be understood as a measure of the heat of activation for one molecule of the substance used in the respiratory process, or of the respiratory catalyst. There is reason to think that the value 11,500 indicates the existence of hydroxyl ion catalysis as the "slow reaction" in such a catenary series as $A \rightarrow B \rightarrow C$, while 16,200 marks a slower aerobic respiration (possibly catalyzed by Fe). See also a later paper by Navez (J. Gen. Physiol. 12: 641-667. 1929) for an excellent study of the relation of the temperature to the respiration of seedlings of *Vicia faba*. This investigator applied the same method of analysis as that used by Crozier and reports the value of 16,250 for μ in experiments in which the temperature was under very exact control.

Another coefficient has been developed by Bělehrádek (Compt. rend. soc. de biol. 95: 1449. 1926; Nature. 118: 117 and 478. 1926; Protoplasma. 7: 232. 1929; Biol. Rev. & Biol. Proc. Camb. Phil. Soc. 5: 30. 1930) who has derived a formula in which the coefficient b does not vary with temperature but does increase with age and is said to be related to the viscosity of the protoplasm. In connection with this formula, however, see Crozier and Stier. J. Gen. Physiol. 10: 501. 1926-27 and Stiles, W. Biol. Rev. & Biol. Proc. Camb. Phil. Soc. 5: 171. 1930 for adverse opinions of this new coefficient.—Ed.

⁶ Bonnier, G. et Mangin. Ann. Sci. Nat. Bot. (6) 17: 210. 1884; 19: 217. 1884.

⁷ Palladin, W. Rev. gén. bot. 11: 241. 1899.

⁸ Iraklionoff. Jahrb. wiss. Bot. 51: 515. 1912; Arb. d. Petersb. Naturforschges. 42: 241. 1911 (Russian).

⁹ Puriewitsch, K. Ann. Sci. Nat. Bot. (8) 1: 1. 1905.

so in the case of succulents, which with lower temperatures effect an incomplete oxidation of sugar and take in a surplus of oxygen which is used to form organic acids. At higher temperature the sugar is burned evenly and the respiratory quotient is 1.¹

The influence of light on respiration. The stimulatory effect of light on the respiration of plant parts containing chlorophyll² is undoubtedly due to the fact that particularly in the light there is a formation of carbohydrates which then serve as material for respiration. The respiration of plants and plant parts without chlorophyll is practically unaffected by light³ or is somewhat retarded.⁴ It must be noticed, however, that all investigations of the influence of light on plant respiration have been carried out in the absence of materials sensitive to light. It would be most interesting to perform experiments with intensive illumination, on the action of catalysts sensitive to light on the respiratory changes. Likewise in the study of the effect of light, the influence of the heating of the material by the rays of light has not always been taken into consideration.⁵ A. Mayer and Deleano⁶ noted a daily periodicity in the rate of respiration under natural conditions. Spoehr⁷ has made the interesting assumption that during the day the respiration of plants is increased by reason of the fact that under the influence of the sun's rays an ionization of the atmospheric oxygen takes place and in consequence thereof autoxidation is instigated in the protoplasm.

The influence of the concentration of oxygen on respiration. Saussure⁸ has pointed out that a reduction of the content of oxygen in the surrounding atmosphere to half the normal concentration has no effect on plant respiration. Wilson⁹ subsequently proved that plants respire normally in the artificial mixture of 0.2 parts atmospheric air and 0.8 parts hydrogen, and indeed showed the same rate of respiration as in ordinary air. Even with an oxygen content of 1% no retardation of plant respiration could be noted. Likewise the ratio $\frac{\text{CO}_2}{\text{O}_2}$ grew larger only with a very low content of oxygen (1-2%).¹⁰ In pure oxygen plants respire more vigorously but are soon exhausted by it.¹¹

¹ Aubert, E. Rev. gén. bot. 4: 203. 1892.

² Borodin, J. Physiological investigations of the respiration of leafy shoots. 1876 (Russian).

³ Maximow, N. Centralbl. f. Bakt. (II) 9: 193. 1902.

⁴ Bonnier et Mangin. Compt. rend. 96: 1075. 1883; 99: 160. 1884; 102: 123. 1886; Idem. Ann. Sci. Nat. Bot. (6) 17: 210. 1884; 18: 293. 1884; 19: 217. 1884; Elfving. Studien über die Einwirkung des Lichtes auf die Pflanze. 1890; Lewschin, A. Beih. z. bot. Centralbl. 23: 54. 1907.

⁵ Waller. New. Phyt. 25: 109. 1926.

⁶ Mayer, A. und Deleano. Zeitschr. f. Bot. 3: 657. 1911.

⁷ Spoehr, H. A. Bot. Gaz. 59: 366. 1916. [This hypothesis has been tested by students of Blackman who have increased the ionization of air by exposure to polonium. Middleton (Ann. Bot. 41: 343-356. 1927) used barley seedlings and Whimster (Ibid. 41: 357-374. 1927) worked with leaves of Pelargonium zonale. Under properly controlled conditions, both workers found that the rate of CO₂-production is appreciably increased by the presence of a high concentration of ions in the air. However, another worker in the same laboratory (de Boer. Ann. Bot. 44: 989-999. 1930) made the same tests with the fungi *Phycomyces Blakesleeanus* and *Polyporus destructor* and reported no effect on the respiration.]

⁸ de Saussure, Th. Mém. soc. phys. de Genève. 6: 552. 1833.

⁹ Wilson. Untersuch. aus d. bot. Inst. Tübingen. 1: 685. 1885.

¹⁰ Johannsen, W. Ibid. 1: 716. 1885; Stich, C. Flora. 74: 1. 1891.

¹¹ Borodin, J. Bot. Zeitschr. 39: 127. 1881; Déhéraïn et Landrin. Compt. rend. 78: 1488. 1874.

The question has also been raised whether a deficiency of oxygen does not exist naturally in the interior of large plant parts because of insufficient aeration. Older workers¹ expressed their opinion that the systematic arrangement of plant organs precludes a deficiency, yet it subsequently appeared that this question is complicated. It was shown that living wood parenchyma cells,² roots,³ and germinating seeds with compact, swollen coats⁴ have an oxygen deficiency under natural conditions. In large fruits the composition of the gas mixture in the internal hollow spaces is not the same as that of the outside air. An analysis of the gas contained in the hollow of a large pumpkin gave the following result: $\text{CO}_2 = 2.52\%$, $\text{O}_2 = 18.29\%$.⁵ In this case, of course, there can be no question of a deficiency of oxygen.⁶ The assumption is not precluded that some plants store quite large amounts of loosely-bound oxygen. This oxygen is set free in an active condition when needed and is used in the oxidation of the respiratory material.

*The influence of CO_2 -concentration respiration.*⁸ Green plants can tolerate high concentrations of CO_2 in the light without injury. However, according to de Saussure, a 40% content of carbon dioxide injures plants. With a high content of CO_2 seed germination is entirely checked,⁷ but is resumed upon restoration of the normal composition of the surrounding atmosphere.⁸ Protoplasmic streaming is also stopped by CO_2 .⁹ It is worthy of note that the respiratory quotient is not altered even in the presence of 40% CO_2 .¹⁰

The influence of nutrient materials on respiration. Borodin¹¹ first showed that carbohydrates, which are formed in the light in green parts of plants, increase the rate of respiration. Subsequently the great importance of the sugars for respiration was established beyond doubt by several investigators. Particularly instructive in this connection are the experiments of Palladin¹² who fed green

¹ Pfeffer, W. Abh. Math.-physik, Kl. d. sächs. Ges. d. Wiss. 15: 449. 1889; Celakowski. Flora. 76: 194. 1892.

² Devaux. Compt. rend. 128: 1346. 1899; Idem. Mém. soc. sci. phys. et nat. Bordeaux. June 15, 1899.

³ Stoklasa, J. und A. Ernest. Jahrb. wiss. Bot. 46: 55. 1909.

⁴ Kostytschew, S. Biochem. Zeitschr. 15: 164. 1908; Physiol.-chemical investigations in plant respiration. 1910 (Russian).

⁵ Devaux, H. Ann. sci. nat. bot. (7) 14: 297. 1891; Rev. gén. bot. 3: 49. 1891. [Langdon and Gailey (Langdon. Jour. Amer. Chem. soc. 39: 149. 1917; Science. 49: 573. 1919; Langdon and Gailey. Bot. Gaz. 70: 230. 1920) report carbon monoxide as a respiratory product in the hollow pneumatocysts of *Nereocystis Luetkeana*. The bladders contain nitrogen, oxygen (15 to 25 %), carbon monoxide (1 to 12 %) and no carbon dioxide.]

⁶ Gerber. (Ann. sci. nat. bot. (8) 4: 1. 1896) attempted to prove that a considerable oxygen deficiency often prevails in the interior of fleshy fruits on account of which alcoholic fermentation is introduced as a normal process in fruits.

⁸ Willaman and Beaumont (Plant Physiol. 3: 45-59. 1928) have reported experiments in which apple twigs, potato tubers and wheat have been exposed to high concentrations of CO_2 while the rate of respiration was being measured. In all cases there is a decrease in the rate, followed by immediate recovery when the CO_2 is removed. Then the rate of respiration proceeds to go above the normal on account of increased permeability of the protoplasm which is due to the action of the CO_2 on the proteins.—Ed.

⁷ Bernard, Cl. Leçons sur les effets des subst. toxiques, p. 200. 1883.

⁸ Kidd, F. Proc. Roy. Soc. London (B) 87: 609. 1914; 89: 136 and 612. 1915.

⁹ Lopriore. Jahrb. wiss. Bot. 28: 571. 1895.

¹⁰ Déhéraïn et Maquenne. Ann. sci. agron. franç. et étrangère. Vol. 12. 1886.

¹¹ Borodin. J. loc. cit.

¹² Palladin, W. Rev. gén. bot. 5: 449. 1893; 6: 201. 1894; 13: 18. 1901; Maige, A. et G. Nicolas. Compt. rend. 147: 139. 1908; Rev. gén. bot. 22: 409. 1910.

and etiolated young leaves of various plants with solutions of sugars and other organic nutrient materials and then measured the rate of respiration of the plants. It was found that leaves which contain a very small supply of carbohydrates in general respire feebly. The application of sugar to such leaves always effected a very appreciable increase in the rate of respiration. The fluctuations of the respiratory quotient as influenced by the percentage composition of the organic nutrients were described above.

The influence of concentrations of various solutions on respiration. For molds and other lower plants growing on solutions of various organic materials, the concentration of the solution is of salient importance. On concentrated sugar solutions the respiration is generally weaker than on dilute solutions.¹ The same rule was confirmed for young leaves of seed-plants when they are artificially nourished with sugar.² The depressent effect of high concentrations of sugar is apparently due to osmotic action. Solutions of mineral salts also produce the same effect.³ In other respects the action of mineral substances on oxygen respiration of plants⁴ is quite a complicated process. Antagonism of the metallic cations plays an important rôle. Protoplasmic poisoning by unbalanced solutions is at once revealed by a reduction in the rate of respiration. Balanced solutions, on the contrary, produce no injurious effect and the rate of respiration remains normal.⁵

The influence of chemical and mechanical stimulating effects on respiration. It is known that the reaction of living protoplasm to various stimulating effects is subject to definite laws. Weak stimulations effect an increased protoplasmic activity which often exceeds that of untreated protoplasm. A somewhat stronger stimulation evokes the same effect. After a certain time, however, there follows a condition of protoplasmic fatigue and the activity then follows that of the normal condition. Very strong stimulations result at once in an appreciable decrease in activity.

The results of stimulation of the living cell may be due to the alterations of the respiratory process. For example, wounding evokes an increased protoplasmic activity. In this case there is a considerable increase in the rate of respiration⁶ and in the heat liberated. Since in this case vigorous growth and formative processes take place, the value of $\frac{\text{CO}_2}{\text{O}_2}$ is lowered.

¹ Kosinski. Jahrb. wiss. Bot. 34: 137. 1902.

² Palladin, W. et Komlew. Rev. gén. bot. 14: 497. 1902.

³ Inman, O. L. J. Gen. Physiol. 3: 533. 1921.

⁴ Krzemieniewski, S. Bull. Acad. Cracovie. 1902; Zaleski, W. und Reinhard. Biochem. Zeitschr. 23: 193. 1909; Lyon, C. J. J. Gen. Physiol. 6: 299. 1924. [For a thorough study of the effects of various concentrations of salts of the heavy metals, see Cook, S. F. J. Gen. Physiol. 8: 575-601. 1925-26. Mayer, A. et Plantefol. Ann. physiol. et de physicochem. 2: 288. 1926 give the action of various electrolytes.]

⁵ Gustafson, F. G. J. Gen. Physiol. 2: 17. 1919-20; Brooks, M. M. Ibid. 3: 337. 1920-21.

⁶ Boehm. Bot. Zeitschr. 45: 671. 1887; Stich, C. Flora. 74: 1. 1891; Smirnof. Rev. gén. bot. 15: 26. 1903; Tscherniaeff. Ber. bot. Ges. 23: 207. 1905; Richards. Ann. Bot. 10: 531. 1896; 11: 29. 1897; Zaleski, W. Ber. bot. Ges. 19: 331. 1901; Dorofeieff. Ibid. 20: 396. 1902; Krasnoselsky, T. Ibid. 24: 134. 1906; Müller. Dansk bot. Arkiv. Vol. 4: 1924; Lutman. Bull. Torrey Bot. Club. 53: 429. 1926.

Chemical stimulation is best evoked by weak poisoning. The strength of the stimulant in this case can be altered as desired by the administration of different amounts of the poison.¹ The effects of toxic substances on the oxygen respiration of plants have not yielded the same results in the experiments of different investigators. In fairly weak narcosis the rate of respiration increases and remains a long time in the stimulated condition, after which it finally returns to the normal condition.² With stronger poisoning the increase in respiration is accompanied by a subsequent decrease.³ Strong poisons applied in sufficiently large quantities effect an immediate marked decrease in respiration.⁴ Under the influence of hydrogen cyanide the output of CO₂ in some cases is entirely suspended without death ensuing. The absorption of oxygen continues to a small extent and after some time returns to normal if the poison is removed.⁵ After a prolonged stay in an oxygen-free medium, the mold *Aspergillus niger* is so badly poisoned by the products of anaerobic metabolism that the gas exchange becomes *nil* although the fungus is not yet killed.⁶

The following table, taken from the extensive monograph by Morkowin,⁷ illustrates the strongest increases in respiratory energy induced by toxic substances.

Plant material	Not stimulated CO ₂ in mg.	Stimulated CO ₂ in mg.	Stimulant
Etiol. leaves of <i>Lupinus luteus</i>	124.9	242.6	Paraldehyde
Etiol. leaves of <i>Vicia Faba</i>	160.4	296.3	Ether
Etiol. leaves of <i>Vicia Faba</i>	189.9	229.5	Pyridine
Etiol. leaves of <i>Vicia Faba</i>	82.5	178.2	Cocaine
Etiol. leaves of <i>Vicia Faba</i>	49.3	89.3	Morphine
Etiol. leaves of <i>Vicia Faba</i>	39.7	105.6	Quinine
Etiol. leaves of <i>Vicia Faba</i>	45.6	97.8	Solanin

Of the usual explanations of toxic action, the following are worthy of note.

Palladin⁸ thought that under the influence of poisons there was a large production of respiratory enzymes within the poisoned cells. According to Palladin, this is a general result of weak poisoning. It is established that enzymatic oxidation and fermentation processes cannot be increased by alkaloids and other stimulants outside of cells. At present the formation of an enzyme outside of living protoplasm is also unknown.

¹ Elfving. Ofvers. af Finska vet. soc. Vol. 28. 1886; Johannsen. Bot. Centralbl. 68: 337. 1896; Morkowin, N. The influence of anaesthetics and poisons on plant respiration. 1901 (Russian); Jacobi, B. Flora. 86: 289. 1899; Brooks, M. M. J. Gen. Physiol. 1: 195. 1918-19; Thomas, H. S. *Ibid.* 1: 203. 1918-19; Ray, G. B. *Ibid.* 5: 469. 1922-23; Gustafson, F. G. *Ibid.* 1: 181. 1918-19.

² Irving, A. Ann. Bot. 25: 1077. 1911; Thoday, D. *Ibid.* 27: 697. 1913; Haas. Bot. Gaz. 67: 347. 1919.

³ Zaleski, W. On the question of the effect of toxic substances on plant respiration. 1907 (Russian).

⁴ Warburg, O. Zeitschr. f. physiol. Chem. 79: 421. 1912.

⁵ Schroeder, H. Jahrb. wiss. Bot. 44: 409. 1907.

⁶ Kostytschew, S. Investigations of the anaerobic respiration of plants. 1907 (Russian).

⁷ Morkowin, N. *loc. cit.*

⁸ Palladin, W. Jahrb. wiss. Bot. 47: 431. 1910.

Warburg¹ has expressed his belief that boundary surface phenomena play an important rôle in the respiratory process. Many poisons are strongly surface active and for that reason interfere violently in the respiratory process (*cf.* pp. 454-455). Both theories are really valuable as working hypotheses.

Chemically indifferent substances can also produce noticeable stimulating effects under certain conditions. Thus Zaleski² has shown that the rate of respiration of bulbs of *Gladiolus* increases considerably after a short stay in pure water. Possibly the temporary withdrawal of oxygen is here to be considered as the cause. Later, attention will be called to the striking increase in respiration after temporary anaerobiosis, the explanation of which agrees with the modern theories of respiration. According to the data of Kostychev, a slightly alkaline reaction of the surrounding solution has a stimulating influence on the respiratory process.³ This agrees with the fact that the enzymatic, biochemical, oxidation processes require a slightly alkaline reaction.^t Lyon⁴ showed that phosphates increase the oxidation processes of plant cells.

The influence of yeast extracts and of sugar solutions fermented by yeast, on the respiration of plants. According to the data of Kostychev and his co-workers,⁵ the oxygen respiration of plants is very strikingly increased by yeast extracts and particularly by sugar solutions fermented by yeast. This matter will engage our attention later in connection with the discussion of alcoholic fermentation.

The increase in rate of respiration under the influence of fermented sugar solutions is entirely unique. Such an increase is not even approximated by the provision of nutrients or even by the action of poisons. The foregoing table of Morkowin shows that the rate of respiration of plants stimulated by poisons reaches at the most 266% of the normal rate (the quinine effect). From fermented solutions there result CO₂-productions which exceed 400-600% of the normal rate for the material used. In examining the following table it is to be taken into consideration that the rate of respiration of control portions (in 5% sugar solution) amounts to 150% of the normal of the wheat embryos (without sugar). The table is an extract taken from Kostychev and Scheloumow.

¹ Warburg, O. *Zeitschr. f. Elektrochem.* 28: 70. 1922; [*Idem.* *Science.* 61: 575-582. 1925; *Biochem. Zeitschr.* 119: 134. 1921].

² Zaleski, W. *loc. cit.*

³ Kostytschew, S. und A. Scheloumow. *Jahrb. wiss. Bot.* 50: 157. 1911; see also Loeb, J. und H. Wasteneys. *Biochem. Zeitschr.* 37: 410. 1911; *Jour. Biol. Chem.* 14: 335, 459, 469 and 517. 1913; 21: 153. 1915. [For the influence of hydrogen ion concentration on respiration, see Gustafson, F. G. *Ibid.* 2: 617-626. 1919-20; and Bode, H. R. *Jahrb. wiss. Bot.* 65: 352-387. 1926. The latter deals only with *Spirogyra* and chiefly on the acid side of neutrality.]

^t It is difficult to reconcile this idea with the increasing evidence that the reaction of protoplasm is on the acid rather than the alkaline side of neutrality (*cf.* Crozier. *Proc. Soc. Exp. Biol. Med.* 21: 58. 1923). On the other hand, the influence of an alkaline reaction may be regarded as more favorable to the catalytic action of iron (Warburg, O. *Science.* 61: 575-582. 1925; Meyerhof und Matsuoka. *Biochem. Zeitschr.* 150: 1-11. 1924).—*Ed.*

⁴ Lyon, C. J. *J. Gen. Physiol.* 10: 599. 1926-27.

⁵ Kostytschew, S. *Biochem. Zeitschr.* 15: 164. 1908; 23: 137. 1909; *Physiol.-chemical investigations of plant respiration.* 1910 (Russian); Kostytschew und Scheloumow. *loc. cit.*; *Ber. bot. Ges.* 31: 422. 1913; Kostytschew, W. Brilliant und A. Scheloumow. *Ibid.* 31: 432. 1913. [Confirmed in part by Bodnar und Hoffner. *Biochem. Zeitschr.* 165: 145-167. 1925.]

CO₂-PRODUCTION OF SOAKED WHEAT EMBRYOS IN MG.

A. In 5% sugar sol.	B. In yeast extracts plus 5% sugar sol.	C. In a fermented 5% sugar solution
37.4	120.8	138.4
63.0	132.0	143.8
22.2	80.6	92.4
26.4	80.2	96.4

This table shows that fermented sugar solutions always effect a larger increase in rate of respiration than yeast extracts plus sugar. From the following data, however, it is apparent that the addition of sugar alone increases the CO₂-production of wheat embryos:

CO₂-PRODUCTION OF SOAKED WHEAT EMBRYOS

A. In water	B. In a 5% sugar solution
86.4 mg.	133.9 mg.

6. RESPIRATION AT THE EXPENSE OF MINERAL SUBSTANCES

It is evident from the foregoing explanations that all combustible substances might be used as respiratory material, if only the living cell is able to oxidize them. Since the physiological significance of respiration is only the release of energy, it is immaterial what processes furnish the kinetic energy. Accordingly we are entirely justified in designating as respiration all the oxidative processes which form the basis of the chemosyntheses of the various bacteria. The sulphur bacteria do not oxidise sugar but hydrogen sulphide and produce not carbon dioxide and water but sulphuric acid. Nitrifying bacteria respire at the expense of ammonia and hydrogen bacteria at the expense of hydrogen. It was shown in Chap. III that there is hardly a true respiration, involving the formation of CO₂, in the case of the nitrifying forms. This is also theoretically conceivable. The simple oxidations of the mineral substances, which are not complicated processes consisting of several stages and which, as reactions of ions, proceed with an immeasurably high velocity, are especially well suited to furnish the generally large amounts of energy which are required for chemosynthesis.

7. THE GENERAL CONCEPT OF FERMENTATIONS

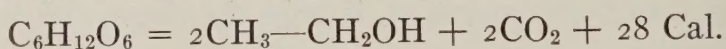
The working energy necessary for the living cell can be produced, of course, not only by processes of oxidation but also by various other exothermal chemical reactions. If such reactions proceed in the absence of oxygen they are called fermentations.

The nature of fermentations was made clear by the great French chemist L. Pasteur. Pasteur said that "fermentation is life without oxygen." The

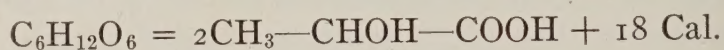
organisms which produce fermentations might actually live in the absence of oxygen because they obtain their working energy not from oxidative processes but from simple cleavage processes and therefore have no true respiration. It was mentioned above that for a long time respiration has been a criterion of life; this view must now be extended inasmuch as *either respiration or fermentation* may supply the working energy.

The processes known to us as *true fermentations* (i.e., cleavage processes which take place without any participation of atmospheric oxygen) can be divided into three large groups: 1. Alcoholic fermentation, 2. Lactic acid fermentation, and 3. Butyric acid fermentation. Pasteur was the first to investigate by reliable methods all these types of fermentation from both the chemical and biological aspects. In addition to the true fermentations, we also recognize the so-called oxidative fermentations which occupy an intermediate position between oxygen respiration and true fermentations. The oxidative fermentations take place only in the presence of oxygen but nevertheless they have some of the important characteristics of true fermentations. The oxidative fermentations include acetic acid fermentation, fermentation of ethyl alcohol to methane, fermentation of polyatomic alcohols to ketone alcohols, and other similar processes. We shall first consider the true fermentations.

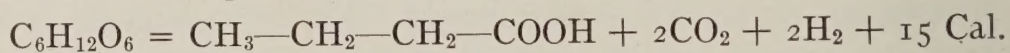
True fermentations are nothing but cleavages of sugar which take place with the formation of heat. Alcoholic fermentation is the decomposition of the simple sugars (hexoses) into ethyl alcohol and carbon dioxide:



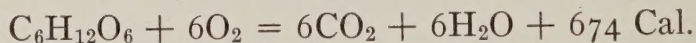
In the case of pure lactic acid fermentation, a hexose molecule breaks into two molecules of lactic acid:



The theoretical equation of butyric acid fermentation reads thus:



It is interesting to compare the foregoing heat productions with that of oxygen respiration:



It is therefore evident that a mol of grape sugar by complete combustion yields just as much energy as 25 mols in the process of alcoholic fermentation, 37 mols in the process of lactic acid fermentation and 42 mols in the process of butyric acid fermentation. This is easily understood. In oxygen respiration all the heat of formation of the grape sugar is set free because CO_2 and H_2O —the products of oxygen respiration—are not combustible. On the contrary, fermentation products are combustible and therefore only a part of the heat of formation of sugar is released during fermentations. Thus the unreleased heat contained in the fermentation products of alcoholic fermentation amounts to 646 Cal., because the heat of combustion of 1 mol of ethyl alcohol equals 325 Cal., the heat of combustion of CO_2 is 0 and the heat of solution of 2 mols of alcohol is equal to 4 Cal.

These figures explain the striking fact that organisms of fermentation always decompose huge quantities of sugar. This is necessary in order to furnish an amount of energy approximately equal to that of the total respiration of sugar. Accordingly, the fermenting organisms often use in one day a quantity of sugar which is several times their own weight.

Each kind of fermentation is produced only by specific microorganisms. Thus we have to do here, just as in chemosyntheses, with biochemical adaptations. The organisms of fermentation actually play an important geochemical rôle on the earth in that they destroy large quantities of organic substances, especially where oxygen is excluded, and thereby make possible the carbon cycle. A very important one in nature is butyric acid fermentation which develops chiefly in the soils and waters where oxidation processes fail because of a deficiency of oxygen.

8. HISTORICAL REVIEW

Alcoholic fermentation and lactic acid fermentation were known even in ancient times because these processes are used to produce alcoholic drinks and the various dairy products. In spite of this, for several centuries it did not occur to any investigators to interpret these fermentations as biological processes. In this respect yeast is really one of the oldest cultivated plants yet man did not realize that he was growing countless generations of microscopically small fungi. There are indications that even Democritus had found how to distill alcohol but alcohol free from water was first obtained in the year 1796 by the Russian chemist Lowitz.

Cagniard-Latour¹ explained the biological nature of yeast. Then it was found that yeasts belong in the group of Ascomycete fungi.² In spite of this it was a long time before correct views of fermentations had gained the ascendancy. Even in the middle of the 19th century the theories of Traube and especially those of Liebig predominated in the field of fermentation. The last-named investigator³ claimed that in the case of their chemical decomposition, various substances undergo peculiar molecular vibrations which were passed on to other substances and produced a cleavage of them. On the basis of this hypothesis, Liebig explained the process of alcoholic fermentation as follows. Fermentation is brought about by a *spontaneous* splitting of protein in the surrounding solution. The molecular vibrations of the protein molecule were said to loosen the sugar molecules and to split them into molecules of alcohol and CO₂. Liebig emphasized that the same process takes place in the presence of yeast cells. According to Liebig's view, living yeast cells effect no alcoholic fermentation. Liebig ascribed a power of fermentation only to dead yeast cells and especially because from dead cells there was a diffusion of the proteins which caused molecular vibrations by their cleavage and thereby brought about the cleavage of sugar. Of course, this theory was not based on experimental

¹ Cagniard-Latour. Compt. rend. 4:905. 1837; Ann. de chim. et de physique. 68:206. 1838.

² Rees, M. Bot. Zeit. 27: 105. 1869; Botanische Untersuchungen über die Alkoholgärungspilze. 1870.

³ Liebig, J. v. Liebigs Ann. d. Chem. 29: 100. 1839.

evidence. Whenever it is thought in recent times¹ that this theory, which was attacked by Pasteur, contains some truth it represents a misunderstanding based on insufficient knowledge of Liebig's original paper.² Traube³ also claimed that alcoholic fermentation is not a biological process and likewise acetic acid fermentation which resembles an oxidation of ethyl alcohol by platinum black, since in the German vinegar industry beech shavings are used as objects with a large amount of surface which accelerates the formation of vinegar.

It was not long before these abstract hypotheses were exploded. The experimental studies of Pasteur, the accuracy of which is a source of astonishment even in our time, gave an entirely different meaning to the mysterious processes of fermentation. Even in his first papers on lactic acid fermentation, Pasteur⁴ developed new ideas which are still valid and which form the basis of the theory of fermentations. Pasteur then turned to alcoholic fermentation and his work on this subject⁵ is really the most magnificent accomplishment in the whole field of the biochemistry and physiology of plants. The new theory of fermentation was strengthened by exemplary, ingenious experiments. Pasteur proved that yeast is a specific organism of fermentation, which lives where oxygen is excluded and has the power to multiply there. Pasteur showed further alcoholic fermentation is impossible in the absence of yeast cells. Liebig and his adherents at first challenged all these facts but later acknowledged them to be correct. The following classical experiment of Pasteur was very important in the disproof of the erroneous views of Liebig and his school. Pasteur added an ammonium salt, as the only source of nitrogen, to a solution of sugar and inoculated it with a minimum amount of yeast, whereupon there was a gradual development of a rapid alcoholic fermentation and the amount of yeast increased considerably, so that the determination of the protein nitrogen proved a marked increase in protein during fermentation. It is evident that alcoholic fermentation is not started by the cleavage of protein.

This experiment was the first proof that mineral substances are indispensable for the lower plants. Of course, such results were made possible only by experiments with pure cultures, since the growth of yeast in impure cultures in the absence of nitrogenous organic substances is usually suppressed by the development of other forms better adapted to purely mineral nitrogen nutrition. Since Pasteur was the first to work out methods for pure cultures, he was able to solve other important problems. For example, he proved that each type of fermentation is brought about by its own specific microbes. In addition, pure cultures

¹ Such assumptions are unfortunately met with even in the fundamental book by **F. Lafar**: *Handbuch d. tech. Mycologie*. 4: 346. 1906.

² Later Liebig, under the influence of Pasteur's impressive demonstrations, changed his original viewpoint and assumed that yeast ferments sugar by forming an enzyme (see below). Since this new standpoint was only a supplement to the biological theory developed by Pasteur, the latter did not contradict Liebig's new ideas. He greeted it rather as a welcome disposition of the long controversy.

³ **Traube, M.** *Poggend. Ann.* 171: 331. 1858.

⁴ **Pasteur, L.** *Compt. rend.* 45: 2 and 913. 1857; 48: 337. 1859; *Ann. de chim. et de physique* (3) 52: 404. 1858.

⁵ **Pasteur, L.** *Ibid.* 58: 323. 1860; *Compt. rend.* 48: 640. 1859; 52: 1260. 1861; 75: 754 and 1054. 1872; *Etudes sur la bière*. 1876.

were an invaluable aid to Pasteur in solving the problem of the spontaneous generation of microorganisms.¹ This was the basis of the further immortal discoveries of Pasteur which revolutionized medicine and saved the lives of millions of people.

At that time, science was not sufficiently mature to accept Pasteur's new ideas without a struggle. The fate of great men who are ahead of their times is for the most part tragic. It is sufficient to point out the great J. R. Mayer who unfortunately came out much "too early" with the first law of thermodynamics. Pasteur also belongs in the same category of great men but he in an exception to the general rule inasmuch as his discoveries were appreciated with a force which disrupted the older science and led to entirely new ideas. This was due entirely to his experimental skill. Actually he did not make a single experimental error; even a slight error would have been fatal for the whole new theory at that time of distrust and suspicion.

In this hard struggle, Pasteur made the surprising discovery of butyric acid fermentation. While the botanists (who regarded Pasteur as a layman in their field of investigation) in particular were questioning the possibility of life without excess to oxygen, Pasteur discovered the causal agent of butyric acid fermentation and found that oxygen is a strong poison for these organisms. Thus it appears not only that life *without* oxygen is possible but that in some cases life *with* access to oxygen is impossible. This remarkable discovery was at first received with scorn and disdain but after some time Pasteur proved the characteristic features of butyric acid fermentation by means of skillful experiments. Finally Brefeld and Traube, two prominent opponents of Pasteur, adopted his ideas. Liebig² was also forced to change his viewpoints repeatedly and at last accepted Pasteur's theory of fermentation in that he assumed that alcoholic fermentation is not started by the supposed cleavage of proteins but by the enzymes contained in the living yeast. Since Pasteur also developed the same views, the years of controversy were ended.³ Pasteur collected most of his studies of fermentation into three famous works: 1. "Investigations of Vinegar," 2. "Investigations of Wine" and 3. "Investigations of Beer." 100 years have passed since this great investigator came into the world and his discoveries still have actual scientific meaning.

9. ALCOHOLIC FERMENTATION

Various fungi of the genus *Saccharomyces* are to be regarded as typical producers of alcoholic fermentation; however, it will be clear from the following that the capacity to produce alcoholic fermentation is contained in most plants and individual plant cells. Some species of fungi of the genera *Oidium*, *Monilia*, *Mucor*, *Allescheria*, and others are also energetic producers of fermentation. Different strains of yeast are not equally strong as producers of fermentation.

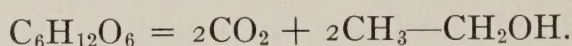
¹ Pasteur, L. Ann. de chim. et de physique (3) 64: 5. 1862.

² Liebig, J. v. Verhandl. d. Bayer. Akad. d. Wiss. 2: 323. 1869; Liebigs Ann. d. Chem. 153: 1 and 137. 1870.

³ Cf. Pasteur, L. Ann. de chim. et de physique (4) 25: 145. 1872; Idem. Etudes sur la bière. 1876.

The nature of alcoholic fermentation was made clear by Pasteur.¹ In addition to the principal products—alcohol and CO₂—there are various by-products including acetaldehyde, acetic acid, glycerine, succinic acid and fusel oils (*loc. cit.*). In addition, there are very small quantities of unidentified esters which account for the so-called aroma of wine and the characteristic taste of beer and are therefore of great practical importance. Succinic acid and fusel oils are products of changes in the amino acids (*cf.* p. 308 ff.) and thus have nothing to do with the actual fermentation. On the contrary, acetaldehyde, acetic acid and glycerine are intermediate products of alcoholic fermentation, of which more will be said later.

Alcoholic fermentation can be represented by the following summary equation:



Not all sugars are fermented by yeast; those capable of fermenting are d-glucose, d-fructose, d-mannose and d-galactose² and, in addition, the disaccharides saccharose (cane sugar) and maltose. It was pointed out recently that maltose itself can be fermented without previous hydrolysis³ but this statement has not been confirmed.⁴ Previous to fermentation, cane sugar is

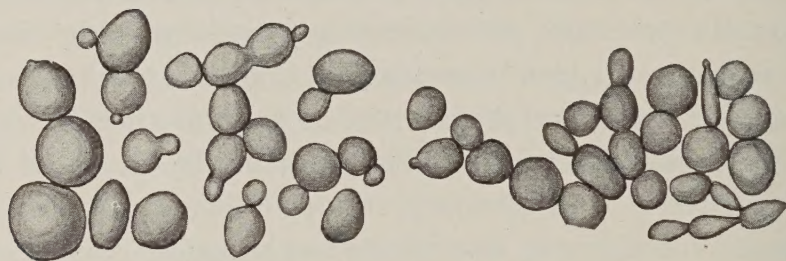


FIG. 41.—Beer yeast, *Saccharomyces Cerevisiae* I. Magnified 650 X. (After Hansen.)

rapidly hydrolyzed by yeast since the latter contains an extremely large amount of invertase. The disaccharides which are not hydrolyzed by yeast (such as milk sugar) are not fermented even though they consist of fermentable components. Simple sugars (monoses) are not fermentable with equal velocities: d-glucose and d-fructose are the normal material for alcoholic fermentation and are vigorously attacked by yeast while d-mannose ferments less well and d-galactose considerably less still. However, yeast in several generations usually becomes accustomed to the fermentation of galactose, inasmuch as this sugar is fermented just as rapidly as grape sugar in parallel experiments.⁵ In a mixture of the two sugars, however, the grape sugar always disappears much faster.⁶

¹ Pasteur, L. *Ann. de chim. et de physique* (3) 58: 323. 1860.

² Concerning the fermentation of the optically active sugars, see Fernbach, Schoen et Mori. *Ann. Inst. Pasteur.* 42: 805. 1928.

³ Willstätter und Steibelt. *Zeitschr. f. physiol. Chem.* 111: 157. 1920.

⁴ Euler, H. v. und K. Josephson. *Ibid.* 120: 42. 1922.

⁵ Dienert, F. *Ann. Inst. Pasteur.* 14: 139. 1900; Harden, A. und R. V. Norris. *Proc. Roy. Soc. London.* 82: 645. 1910; Euler, H. v., Laurin und Petterson. *Biochem. Zeitschr.* 114: 277. 1921.

⁶ Willstätter, R. und H. Sobotka. *Zeitschr. f. physiol. Chem.* 123: 164, 170 and 176. 1922. According to the statements of these workers, even α -glucose is more easily fermented than β -glucose. Such is the importance of the spatial arrangement of atoms in the sugar molecule.

This important practical knowledge shows that spatial isomerism plays a prominent rôle in the process of fermentation. It has been stated that ethyl alcohol is formed in small quantities by the treatment of sugar solutions with alkalies and without the aid of fermenting fungi;¹ still this output of alcohol is so minute that it need not be considered for theoretical explanations. For the present we must consider that so far there has been no artificial alcoholic fermentation produced by purely chemical means. The hypothesis is not improbable that the first stages of alcoholic fermentation will not be explained until we have become intimate with the true nature of spatial isomerism.

The development of heat during alcoholic fermentation is generally known, since fermented solutions heat up in the course of the process. The rise in temperature may be easily observed in a Dewar flask. Direct experimental determinations of the heat produced by alcoholic fermentation have given a value of 24 Cal. per mol of grape sugar,² which agrees with the theoretical calculations (*cf.* p. 396). Of course, a suitable supply of nitrogen and mineral nutrients is necessary for the good development of yeast and its capacity for fermentation. Yeast assimilates nitrogen best in the form of peptones or amino acids.³ It has been pointed out repeatedly that the formation of enzymes of fermentation depends primarily on the composition and amount of the nitrogenous nutrient materials⁴ but this hypothesis has not been confirmed.⁵ The conversions of the nitrogenous substances in the normal course of the fermentation of sugar consist in the gradual decomposition of the proteins in the yeast cells into amino acids, but the latter change to fusel oils if the nitrogen supply is insufficient. This process was considered in Chap. VI.

10. THE SPONTANEOUS FERMENTATION OF YEAST

When there is a deficiency of material for fermentation, the glycogen stored in the yeast cells starts to ferment; it is remarkable that the gums of yeast are not thereby affected.⁶ At the same time there is a vigorous autolysis of yeast cells which eventually leads to the death of the yeast. This fermentation in the starving condition is called spontaneous fermentation. The energy with which the enzymes of the yeast cells work during starvation is evident from the fact that all the glycogen in the starving cells disappears in 3 to 4 hours.⁷ Conversely, at high concentration of sugar there is a noticeable enzymatic synthesis of glycogen which supposedly takes place outside the living cells.⁸ The chemical aspects of the cleavage of carbohydrates is exactly the same in spontaneous

¹ Duclaux, E. *Ann. Inst. Pasteur.* 7: 751. 1893; 10: 168. 1896; Buchner, E. und J. Meisenheimer. *Ber. chem. Ges.* 38: 626. 1905.

² Rubner, M. *Arch. f. Hyg.* 49: 355. 1904; and others.

³ Lindner, Rölke und Hoffmann. *Wochenschr. f. Brauer.* 22: 528. 1905; Lindner und Stockhausen. *Ibid.* 23: 519. 1916; Rubner, M. *Die Ernährungsphysiologie der Hefezelle bei alkoholischer Gärung.* 1913; and others.

⁴ Iwanowski, D. *Investigations in alcoholic fermentation.* 1894 (Russian); *Idem.* *Centralbl. f. Bakt. (II)* 10: 151, 180 and 209. 1903.

⁵ Richter, A. v. *Centralbl. f. Bakt. (II)* 8: 787. 1902; *Idem.* *Critical contributions to the theory of fermentation.* 1903 (Russian).

⁶ Euler, H. v. und K. Myrbäck. *Zeitschr. f. physiol. Chem.* 47: 554. 1904.

⁷ Buchner, E. und Mitscherlich. *Zeitschr. f. physiol. Chem.* 47: 554. 1904.

⁸ Harden, A. and Young. *Biochem. Jour.* 7: 630. 1913.

fermentation as in the fermentation of sugar.¹ In view of the ease with which the glycogen of yeast is formed or split up, Grüss² assumes that in both cases all the fermented sugar is temporarily changed to glycogen. This hypothesis has not yet been proved. A simultaneous and extremely vigorous cleavage of protein which goes as far as the change of about 90% protein nitrogen into crystalline nitrogenous substances³ is a symptom of spontaneous fermentation. In addition, there are other interesting processes in the case of spontaneous fermentation, such as a cleavage or inactivation of part of the enzymes of yeast by other enzymes.⁴ It is therefore to be regretted that only the older investigations⁵ of the chemical processes apply in the case of autolysis of yeast. All external factors which produce a plasmolysis of the yeast cells, increase the rate of spontaneous fermentation and the related autolytic processes.⁶

II. CELL-FREE ALCOHOLIC FERMENTATION

E. Buchner⁷ has reported experiments which were said to prove that alcoholic fermentation is also possible without yeast cells and that it is induced by an enzyme. This enzyme was named zymase. At first the following methods were recommended for its preparation. The yeast cells are macerated with quartz sand, fine fragments of glass or diatomaceous earth to form a sticky, homogeneous mass which is then pressed in a hydraulic press at 300–400 atmospheres.⁸ The filtered juice is the material for experiments on cell-free fermentation. Now the pressed juice is seldom prepared because another method proved more convenient. The yeast is dried at a moderate temperature (about 30°), then extracted with distilled water at 25–30° and filtered.⁹ The clear filtrate, the so-called maceration juice, unlike the pressed juice contains no glycogen and therefore shows no spontaneous fermentation but it still has the capacity to ferment sugar that is added to it. A third method is to wash the yeast with acetone which leaves the enzymes of the cells in an active condition.¹⁰ These preparations have been introduced into the trade as “zymin” or “hefanol.”

At present some of the evidence for the existence of enzymatic fermentation must be regarded as antiquated; hence, cell-free fermentation is to be considered as not proved beyond doubt. From theoretical standpoints, the following is not clear: 1. The rate of fermentation of yeast juice is not proportional to that

¹ Palladin, W. und Kostytschew. *Zeitschr. f. physiol. Chem.* 48: 214. 1906; and others.

² Grüss. *Zeitschr. f. ges. Brauwesen*, p. 27. 1904; Euler, H. v. *Zeitschr. f. physiol. Chem.* 89: 337. 1914.

³ Kostytschew, S. und W. Brilliant. *Zeitschr. f. physiol. Chem.* 91: 372. 1914; *Idem.* *Mitt. d. Akad. d. Wiss. in Petersburg*, p. 953. 1916 (Russian).

⁴ Buchner, E., H. Buchner und M. Hahn. *Die Zymasegärung.* 1903; Grigoriew und Gromow. *Zeitschr. f. physiol. Chem.* 42: 299. 1904; and others.

⁵ Salkowski, E. *Zeitschr. f. physiol. Chem.* 13: 506. 1889; Kutscher, F. *Ibid.* 32: 59. 1901; Buchner, E., H. Buchner und M. Hahn. *Die Zymasegärung.* 1903.

⁶ Harden, A. and Paine. *Proc. Chem. Soc.* 27: 103. 1911; Harden, A. *Proc. Roy. Soc. London (B)* 84: 488. 1912.

⁷ Buchner, E. *Ber. chem. Ges.* 30: 1112. 1897; Buchner, E., H. Buchner und M. Hahn. *Die Zymasegärung.* 1903.

⁸ Buchner, Buchner und Hahn. *loc. cit.*

⁹ Lebedew, A. v. *Compt. rend.* 152: 49 and 1129. 1911; *Zeitschr. f. physiol. Chem.* 73: 447. 1911.

¹⁰ Albert, Buchner und Rapp. *Ber. chem. Ges.* 35: 2376. 1902; *Idem.* *Zymasegärung.* 1903.

of the yeast from which the juice is prepared. Recent experiments to explain this inconsistency¹ are inadequate. 2. As a rule, enzyme actions proceed very rapidly and start at full strength. The fermentation of yeast juice is not only extremely slight but also of long duration. Not infrequently there is evidence of an "induction period" which enzymes do not have. 3. The modern theories of fermentation make it necessary to assume that zymase represents a complex of several enzymes. If this is so, in the first place, a regulation of all the enzyme actions in a structure-less medium must be presupposed—a condition which contradicts all our conceptions of regulatory phenomena. Secondly, it is inconceivable that the enzymes are not mixed by the grinding of the yeast with diatomaceous earth and by analogous treatments. According to Willstätter (p. 44), the best means of separating enzymes is selective adsorption. Separation would at once result in an alteration of the chemical course of the fermentation.

The objections to the experimental side of experiments in "cell-free fermentation" are of a two-fold nature. In the first place, the methods for all these experiments are found to be inadequate; in many cases the presence of living yeast cells and bacteria, capable of producing fermentation, can be demonstrated and the incompleteness of the sterilization has been proved.² Secondly, the fermentation of yeast juice shows many of the characteristics of a biological process; the fermentation is stimulated by small amounts of poisons and may be passed on from juice to juice.³ Zymase also differs from other enzymes in diffusion behavior.⁴

New investigations in this field are therefore necessary and it is essential to revise the present views which are obsolete in many respects. Should it be found that cell-free fermentation does proceed under strictly aseptic conditions, it will be necessary to find new terms with reference to the components of the cell. The term "enzyme" is now so sharply defined that it cannot be applied in the case of all material transformations which take place after the death of the cells without further enzyme action.

12. THE INFLUENCE OF EXTERNAL FACTORS ON ALCOHOLIC FERMENTATION

Alcoholic fermentation is dependent on external influences in a way analogous to that of oxygen respiration. It will be shown later that this is not an accidental analogy. The latest discovery of the rhythmic nature of alcoholic fermentation is interesting; the process is not uniform but proceeds by stages.⁵ Short periods of increased rate of fermentation follow brief periods of paralysis

¹ Meyerhof, O. *Biochem. Zeitschr.* 203: 252. 1928.

² Abderhalden, E. und A. Fodor. *Fermentforschung.* 5: 128. 1921; Kostytschew, S., G. Medwedew und H. Kardo-Sysoiewa. *Zeitschr. f. physiol. Chem.* 168: 244. 1927; Kostytschew, S. und V. Faermann. *Ibid.* 176: 46. 1928; Kostytschew, S. und O. Schulgina. *Ibid.* 182: 50. 1929. [The exceptions which Lebedew has taken to the work reported in these papers (cf. Lebedew, A. v. *Biochem. Zeitschr.* 214: 488. 1929) may be noted but the evidence seems to favor the new views. For a brief reply to Lebedew's paper see Kostytschew, S. *Ibid.* 218: 402. 1930.]

³ Kostytschew, S. und A. Chomitsch. *Ibid.* 176: 55. 1928; Kostytschew, S. und Berg. An unpublished paper.

⁴ Kostytschew, S. und S. Klupt. An unpublished paper.

⁵ Köhler, E. *Biochem. Zeitschr.* 106: 191. 1920; 108: 235. 1920; 110: 128. 1920.

(the measurements were taken every 10 seconds). There are various possible explanations of this phenomenon; of course, they are all purely hypothetical as long as the chemical aspects of fermentation are not made clear.

The influence of temperature on alcoholic fermentation is a complicated matter. We must distinguish sharply between the direct influence of temperature on the chemical reaction of the fermentation of sugar and the indirect influence. The latter can happen because the multiplication of the yeast cells depends to a large extent on the temperature. Of course, a larger amount of yeast ferments sugar more rapidly. Analogous considerations apply to all investigations of the influence of external factors on yeast fermentation. If one wishes to investigate only the chemical reaction *sensu stricto*, a very short experiment is performed with considerable quantities of yeast. Under these conditions the multiplication of yeast is practically negligible.

The upper temperature limit for yeast fermentation lies at about 50° and the lower limit at about 0°. In practice a temperature of 25–35° is the most favorable. The temperature coefficient of fermentation¹ is about 2, which agrees with van't Hoff's law (p. 25).

The Influence of Light on Alcoholic Fermentation.—Alcoholic fermentation, like oxygen respiration, is somewhat retarded by bright light, due probably to a reduction of the multiplication of yeast.² Much more interesting is the latest indication that the ultra-violet rays have an extraordinary stimulating effect on the rate of fermentation.³ After 6 hours of illumination the formation of CO₂ is 9 times greater and after 23 hours of illumination it is 23 times greater than that of unilluminated controls.

The Influence of Sugar and Its Fermentation Products.—Living yeast can ferment very concentrated solutions of sugar (up to 60%).⁴ Invertase is more strongly retarded by high concentrations of sugar than zymase so the limiting value of the concentration of cane sugar is lower than for grape sugar.⁵ In practice, 25% solutions are often fermented. The use of more concentrated solutions is not desirable because in this case a total fermentation is prevented by too high a content of alcohol. Between 5% and 20% there is practically no influence of the change in concentration on the fermentation.⁶

Yeast is less sensitive to ethyl alcohol than most other plants. An alcohol content of 3% does not retard the multiplication of yeast.⁷ The limiting concentration with which fermentation is still possible is usually 10 to 14%.⁸

¹ Herzog. Zeitschr. f. physiol. Chem. 37: 149. 1902; Slator, A. Wochenschr. f. Brauerei. 28: 141. 1911.

² Kny. Ber. bot. Ges. 2: 129. 1884; Lohmann. Diss. Rostock. 1896; Lubimenko, W. Compt. rend. 154: 226. 1911.

³ Lindner, P. Wochenschr. f. Brauerei. 39: 166. 1922.

⁴ Laurent. Ann. Inst. Pasteur. 2: 603. 1888.

⁵ Bokorny. Chem. Zeit. Vol. 27. 1903; Idem. Centralbl. f. Bakt. (II) 12: 119. 1904.

⁶ Brown. Jour. Chem. Soc. (London) 61: 369. 1892; cf. also Dumas. Ann. de chim. et de physique (5) 3: 57. 1874.

⁷ Brown, A. J. Jour. Chem. Soc. (London). 87: 1395. 1905; Hayduck, M. Zeitschr. f. Spiritusind. 4: 173 and 341. 1881; 5: 183. 1882.

⁸ Hayduck, M. loc. cit.; Brefeld, O. Landwirt. Jahrb. 5: 281. 1876; Müller-Thurgau, H. 3 Jahresber. d. Versuch. Wädensweil. Vol. 73. 1892–93; Yabe, K. Bull. Coll. Agric. Tokyo. 2: 219. 1896 gives a growth limit of 24% alcohol for a Japanese strain of yeast.

In concentrated solutions of sugar, yeast is relatively more sensitive to the high content of alcohol. An increase in temperature also increases the injurious effect of alcohol.¹

Carbon dioxide likewise exerts only a weak effect on alcoholic fermentation; it often continues in a solution saturated with CO₂. Still it is desirable in the brewing industry to drive the carbon dioxide from the solution by means of a current of air, because this facilitates fermentation and the reproduction of the yeast.² On the other, hand, alcoholic fermentation is not stopped even by a CO₂ pressure of several atmospheres.³ According to recent statements,⁴ alcoholic fermentation is possible at a CO₂ pressure of 60 atmospheres and therefore in the presence of liquid carbon dioxide.

The Influence of Mechanical and Chemical Irritant Effects on Alcoholic Fermentation.—In the brewing industry the passage of a current of air through the fermenting wort is customary not only because the CO₂ is thereby removed and the growth of the yeast helped, but also because the yeast is set into a slow gliding motion and the nutrient substances are evenly distributed. A powerful and continuous shaking, however, retards fermentation.⁵

In the pH range 4–8.5, the velocity of yeast fermentation is constant.⁶ Outside of this range the rate of fermentation is lower. We must distinguish here between the direct influence of pH on the fermentation process in the narrow sense and the indirect influence on the multiplication of the yeast. The growth of yeast is furthered by a weakly-acid reaction. In the course of fermentation the solution becomes more and more acid, since yeast produces various organic acids (primarily succinic and acetic acid). The following table of Lüers⁷ shows that the acid reaction of the solution reaches a high value in the case of fermentation under certain conditions.

Fermentation at 8° in hours	Hydrogen ion concentration
0	3.39×10^{-6}
25	3.24×10^{-5}
50	4.37×10^{-4}
89	1.29×10^{-3}
160	1.86×10^{-3}

The nature of the effect of the acid reaction on the alcoholic fermentation has not yet been made clear.⁸ Different acids do not stop fermentation at the

¹ Müller-Thurgau, H. Ber. d. Generalvers. d. Weinbau-Vereins. p. 50. 1884.

² Foth. Wochenschr. f. Brauerei. 4: 73. 1887.

³ Cerles et Cochin. Compt. rend. soc. de biol. (8) 1: 639. 1884; Foth. *loc. cit.*; Evans. Jour. Fed. Inst. Brewing. 4: 249. 1898; Matthews, C. G. Wochenschr. f. Brauerei. 4: 380. 1887, ref.

⁴ Kolkwitz, R. Ber. bot. Ges. 39: 219. 1921.

⁵ "Zymasegärung." 1903.

⁶ Hägglund, E. A. Söderblom und B. Froberg. Biochem. Zeitschr. 169: 200. 1926.

⁷ Lüers. Zeitschr. f. ges. Brauwesen. 37: 79. 1914.

⁸ Paul, Birstein und Reuss. Biochem. Zeitschr. 29: 202. 1910; Johannesohn. *Ibid.* 47: 97. 1912.

same concentration of hydrogen ions.¹ At pH 8 the molecular ratio of alcohol to CO₂ remains normal;² with a more alkaline reaction there are modifications of the chemical nature of the fermentation which will be considered later.

Salts of the heavy metals and organic poisons have analogous effects on respiration and fermentation; very small amounts stimulate fermentation but large quantities exert an immediate retarding effect.³ Phosphates and salts of arsenic⁴ stimulate fermentation. It is remarkable that only the cell-free fermentation is strongly stimulated by phosphate while living yeast is more indifferent to phosphates. It is known that phosphates and sugar form the hexose phosphate necessary for fermentation. In addition, it has been thought that phosphates aid in the formation of the insufficiently studied co-enzyme of fermentation.

Fermentation is accelerated by aqueous extracts of living and killed yeast. The action of a co-enzyme also appears in this case.⁵ The hypothesis is also not precluded that fermentation is stimulated by vitamins in the extract.⁶ There are direct statements concerning the strong stimulation of alcoholic fermentation by vitamin B, in the presence of sufficient amounts of the "co-enzyme."⁷ It has recently been shown⁸ that finely divided animal charcoal stimulates alcoholic fermentation even more than phosphates and hexose phosphate. It is remarkable that talc, kaolin and diatomaceous earth have no such effect. The action of animal charcoal is also limited not only to the quantitative acceleration of the cleavage of sugar but it also produces a formation of new products. This will be considered in detail later. Some organic substances, especially pyroracemic acid and acetaldehyde, which are probably intermediate products of alcoholic fermentation, likewise increase the rate of fermentation.⁹

13. THE INFLUENCE OF OXYGEN ON ALCOHOLIC FERMENTATION

This must be considered separately because we are dealing here with a phenomenon which is closely connected with the modern theories of fermenta-

¹ Hägglund, E. Hefe und Gärung in ihrer Abhängigkeit von Wasserstoff und Hydroxylionen. 1914.

² Euler, H. v. und O. Svanberg. Zeitschr. f. physiol. Chem. 105: 187. 1919.

³ Hüne. Centralbl. f. Bakt. (I) 48: 135. 1909; Brown, F. Ibid. (II) 31: 185. 1911; Bokorny. Biochem. Zeitschr. 50: 1. 1913; Kayser, E. et Marchand. Compt. rend. 144: 574 and 714. 1907; 152: 1279. 1911; Nathan, L. Centralbl. f. Bakt. (II) 14: 289. 1905; Koch. Ibid. 31: 175. 1911; Euler, H. v. und Sahlén. Zeitschr. f. Gärungsphysiol. 3: 225. 1913; and others.

⁴ "Zymasegärung." 1903; Buchner, E. und Antoni. Zeitschr. f. physiol. Chem. 46: 136. 1905; Harden, A. and Young. Proc. Roy. Soc. London. 77: 405. 1906; 78: 369. 1906; 80: 299. 1908; 83: 451. 1911; Idem. Centralbl. f. Bakt. (II) 26: 178. 1910; Idem. Proc. Chem. Soc. 22: 283. 1906.

⁵ Harden, A. and Young. *loc. cit.*; Wochenschr. f. Brauerei. 22: 712. 1905.

⁶ Abderhalden, E. und H. Schaumann. Fermentforschung. 2: 120. 1918; 3: 44. 1919. These workers are correct in demanding that a revision be undertaken of the present conceptions of the mysterious "co-enzyme." [In this connection see Raymond, A. L. Jour. Biol. Chem. 79: 637-648. 1928 who finds that this unknown substance has a "complement enzyme action" in connection with the enzyme which hydrolyzes the hexose phosphate.] Kluyver und Struyk. Proc. Kon. Akad. Wet. Amsterdam. Vol. 30, no. 5. 1927; Biochem. Zeitschr. 201: 212. 1928. On this point *cf.* p. 48.

⁷ Kurono, K. Jour. Coll. Agric. Tokyo. 5: 305. 1915; Euler, H. v. und S. Karlson. Biochem. Zeitschr. 130: 550. 1922.

⁸ Abderhalden, E. Fermentforschung. 5: 89, 110 and 255. 1921; Abderhalden und Fodor. Ibid. 5: 138. 1921; Abderhalden und Wertheimer. Ibid. 6: 1. 1922; Abderhalden und Glaubach. Ibid. 6: 143. 1922; Abderhalden, E. Ibid. 6: 149 and 162. 1922.

⁹ Oppenheimer, M. Zeitschr. f. physiol. Chem. 93: 235. 1914; Harden, A. and F. R. Henley. Biochem. Jour. 14: 642. 1920; 15: 175. 1921; Neuberg, C. Biochem. Zeitschr. 86: 145. 1918; Neuberg, C. und M. Sandberg. Ibid. 126: 153. 1921; and others.

tion and respiration. At first glance it appears that oxygen respiration is superfluous for yeast cells because they obtain their working energy from the alcoholic fermentation. For various reasons, however, Pasteur concluded that yeast respire normally when oxygen is available and uses alcoholic fermentation as the source of energy only when oxygen is excluded.

Pasteur's first experiments¹ showed that when oxygen is available yeast consumes less sugar than when oxygen is excluded. In addition, it appeared that oxygen generally favors the growth and reproduction of yeast cells.

Later this question became involved because different workers obtained divergent results. For example, it was pointed out that alcoholic fermentation is not retarded in the presence of oxygen but rather it is accelerated.² In these investigations, however, the influence of oxygen on the multiplication of the yeast was not taken into consideration. It is evident that an increase in the amount of yeast gives a greater production of alcohol; this only an indirect effect of atmospheric oxygen (*cf.* p. 404 and 405).

On the contrary, other investigators claimed that alcoholic fermentation is retarded by the presence of oxygen.³ These statements also proved to be involved.⁴ Finally the view was expressed⁵ that yeast splits equal quantities of sugar, whether oxygen is available or not. This result was interpreted in favor of the hypothesis that alcoholic fermentation is independent of atmospheric oxygen. Yeast is said to get its working energy by the process of alcoholic fermentation both with and without oxygen.

If we take into consideration the quantities of energy set free by oxygen respiration on the one hand and by alcoholic fermentation on the other (see p. 396), we must conclude that the foregoing idea is unproved so long as it has not been measured *as to which kind* of sugar is broken down with oxygen available. We know that 1 mol of grape sugar by total combustion furnishes 25 times as much energy in comparison with the output of alcoholic fermentation. So if only one-twenty-fifth of the vanished sugar is burned in the process of respiration, it is fair to conclude that yeast gets *half* its working energy from oxygen respiration and thus is not entirely indifferent to atmospheric oxygen.

By simultaneous determinations of the sugar consumed and of the fermentation products (*i.e.*, alcohol and carbon dioxide), the quantities of sugar respired and fermented can be measured in the following manner. From the equation for alcoholic fermentation and the data of the numerous, accurate analyses by Pasteur and subsequent workers, it has been established that 100–102 parts by weight of alcohol to every 100 parts by weight of CO₂ are formed by the total fermentation of sugar. We can therefore assume that the amount of CO₂

¹ Pasteur, L. Compt. rend. 52: 1260. 1861; *Idem.* Etudes sur la bière, p. 243. 1876.

² Nägeli, C. v. Theorie der Gärung, p. 18. 1879; Brown, A. Jour. Chem. Soc. (London) 1: 369. 1892.

³ Hoppe-Seyler. Über die Einwirkung des Sauerstoff auf Gärungen. 1881; Pedersen. Medd. frå Carlsberg Lab. 1: 72. 1878; Hansen. *Ibid.* 2: 133. 1879; Chudiakow, N. Landwirt. Jahrb. 23: 391. 1894.

⁴ *Cf.* Rapp, R. Ber. chem. Ges. 20: 1983. 1896; Buchner, H. und R. Rapp. *Ibid.* 29: 1983. 1896; Buchner, H. und R. Rapp. Zeitschr. f. Biol. 37: 82. 1899.

⁵ Mayer, A. Ber. chem. Ges. 13: 1163. 1880; *Idem.* Landwirt. Veruchs-Stationen. 25: 301. 1880; Iwanowsky, D. Investigations in alcoholic fermentation. 1894 (Russian).

equivalent to the total output of alcohol is formed by alcoholic fermentation, while the surplus CO_2 is due to oxygen respiration.

The first experiments carried out according to this method gave fluctuating results,¹ in that 5–25% of the sugar was traced to oxygen respiration. It must be made certain in such experiments that there is free access to oxygen and this was true only of more recent investigations.² From them it has been found that with perfect aeration, about a third of the carbon dioxide formed comes from oxygen respiration. The following figures calculated by the writer of this book from the analyses by H. Buchner and Rapp indicate the value of $\frac{\text{CO}_2}{\text{C}_2\text{H}_5\text{OH}}$ for both complete aeration and deficiency of oxygen. Of course, these figures refer to alcoholic fermentation of living yeast cells.

$\frac{\text{CO}_2}{\text{C}_2\text{H}_5\text{OH}}$ good aeration	$\frac{\text{CO}_2}{\text{C}_2\text{H}_5\text{OH}}$ deficiency of oxygen
100:66	100:105
100:68	100:108
100:67	100: 97
100:59	100: 90
100:68	100:100
100:66	100: 97
100:65	100:107

It is evident that with good aeration the oxygen respiration of yeast yields considerably more energy than the process of fermentation. It seems then that alcoholic fermentation is not physiologically important for yeast when oxygen is available. This question will be considered in more detail on subsequent pages; for the present it suffices to emphasize the fact that, in accord with Pasteur's theory, all the working energy of yeast cells can be furnished by the process of oxygen respiration.³

The question of the influence of oxygen on alcoholic fermentation has been handled here so completely because it is theoretically very important, especially for considerations of the mechanism of oxygen respiration (see below). The practical brewing business has long taken into account that a considerable amount of sugar is burned by yeast with an abundant supply of oxygen and so does not change to alcohol. When pressed yeast is the principal product of the operation, plenty of air is added to hasten the reproduction of the yeast but if ethyl alcohol is the chief product the entry of air must be limited so that most

¹ Giltay und Aberson. Jahrb. wiss. Bot. 26: 543. 1894.

² Buchner, H. und R. Rapp. *loc. cit.*; Idem. "Zymasegärung." 1903; Kostytschew, S. und P. Eliasberg. Zeitschr. f. physiol. Chem. III: 141. 1920. Iwanowsky also worked with good aeration (*loc. cit.*) but he made no simultaneous determinations of CO_2 and alcohol; therefore his experiments are insufficient for the solution of this question.

³ According to Meyerhof. Biochem. Zeitschr. 162: 43. 1925, the oxidation of 1 molecule of sugar provides for the fermentation of 4 to 6 other molecules of sugar.

of the carbon dioxide escapes from the solution while the respiration of the yeast is not very great.

Under certain conditions, the atmospheric oxygen itself also acts as a hydrogen acceptor (see below) in the process of fermentation. For example, some substances are used by yeast when oxygen is freely admitted which are not fermented when oxygen is excluded. Lactic acid,¹ mannite and glycerine² belong in this category. It can hardly be doubted that all these substances change to compounds which can be attacked by yeast, through the removal of two atoms of hydrogen. Under these conditions lactic acid becomes pyroracemic acid, glycerine changes to dioxyacetone (or directly to a hexose after a condensation of the unsaturated radicals left by the removal of hydrogen) and mannite to fructose.

14. THE ALCOHOLIC FERMENTATION OF THE MUCORACEAE

The behavior of the organisms of fermentation becomes more evident if fungi, other than yeast, which can cause fermentation are taken into consideration. Among these, various members of the *Mucoraceae* are particularly interesting. In this family we encounter both energetic agents of fermentation and typical aerobic fungi; the two categories are linked by a series of intermediate species. Good agents of fermentation are *Mucor circinelloides*, *M. javanicus* and *M. erectus*. A somewhat weaker fermentation is produced by *M. Rouxii*, *M. racemosus*, *Rhizopus tonkinensis* and *Rhizopus japonicus*. Still weaker in action are *M. spinosus*, *M. piriformis*, *M. hiemalis*, *M. fragilis*, and others. *Mucor Mucedo* and *Rhizopus nigricans* (*M. stolonifer*) are very weak producers of fermentation. The morphology of these fungi corresponds to their physiological behavior. Weak agents of fermentation like *M. Mucedo* and *Rhizopus nigricans* form a typical mycelium of connected hyphae. On the contrary, active agents of fermentation develop at least in part in the form of Mucor yeasts which strongly resemble the true Saccharomycetes. As the result of the formation of heavy, yeast-like structures, the fungus mat of these fungi is always immersed in the liquid.

Various Mucors could be used as good illustrations of Pasteur's sentence, "Fermentation is life without oxygen." It has been found³ that *Mucor racemosus*, a strong promoter of fermentation, can develop with oxygen entirely excluded. *Mucor Mucedo* is unable to develop without oxygen but mature fungus mats can live for a long time with oxygen excluded. As for *Rhizopus nigricans*, this species, even in the form of a prepared fungus mat, can only live a short time with oxygen excluded. However, it is noteworthy that all three of these species of Mucor in the presence of oxygen exhibit not only oxygen respiration but also alcoholic fermentation.⁴ With good aeration,

¹ Fürth, O. und F. Lieben. Biochem. Zeitschr. 128: 144. 1922; 132: 165. 1922; Myrbäck, K. und B. Everitt. Zeitschr. f. physiol. Chem. 139: 272. 1924.

² Kostytschew, S. und V. Faermann. Zeitschr. f. physiol. Chem. 173: 72. 1928.

³ Kostytschew, S. Centralbl. f. Bakt. (II) 13: 490. 1904.

⁴ Kostytschew, S. und P. Eliasberg. Zeitschr. f. physiol. Chem. 111: 141. 1920.

Rhizopus nigricans also forms alcohol¹ although unquestionably this fungus cannot grow at the expense of alcoholic fermentation. It was found in this connection that the morphologically identical sexual + and - strains of the *Mucors* are not equally good agents of fermentation and differ from each other within a given species in other physiological characteristics.²

The behavior of the *Mucoraceae* in the case of fermentation is somewhat different from that of yeast. The former, like other molds, are nourished satisfactorily with ammonium salts and are able to grow in strongly acid solutions. They are also more sensitive to high concentrations of alcohol than the *Saccharomycetes*. In the case of fermentation by *Mucors*, the limiting concentration of alcohol does not exceed 7%; numerous studies by various investigators have given the following average maximum yields:³

Fungus ¹	Alcohol in %	Fungus	Alcohol in %
<i>Mucor Mucedo</i>	1	<i>Mucor alternans</i>	3-3.5
<i>Rhizopus nigricans</i>	1	<i>Mucor Rouxii</i>	3-4
<i>Rhizopus chinensis</i>	1-2	<i>Mucor javanicus</i>	4
<i>Rhizopus tonkinensis</i>	1-2	<i>Mucor circinelloides</i>	4-5
<i>Mucor racemosus</i>	3	<i>Rhizopus japonicus</i>	4-5

¹ This table is taken from the treatise by C. Wehmer in Lafar's Handbuch d. tech. Mykologie. 4: 519. 1905-1907.

Unlike yeast, the *Mucoraceae* can feed on various organic substances; however, alcoholic fermentation takes place in abundance only on the same sugar solutions which are fermented by yeast. Cane sugar is inverted and fermented only by the - strain of *Mucor racemosus*.⁴ On the contrary, some *Mucors* can change starch to sugar; especially strong diastatic properties are shown by the Eastern Asiatic species *Mucor Rouxii*.⁵ *Rhizopus Oryzae*, *Chlamydomucor Oryzae* and a few other species are also diastatic.⁶ This property of these *Mucors* is useful for commercial purposes. Potato mash is fermented directly to alcohol without treatment with malt by a mixed culture of *Saccharomycetes* and diastatically active *Mucoraceae*. The fungus *Monilia*, which promotes fermentation, also fails to form a true mycelium and the fungus culture always consists in part of a yeast-like formation. *Monilia candida*

¹ Wehmer has already established the formation of alcohol in the presence of air by the most effective agents of fermentation of the *Mucoraceae*. Cf. Wehmer, C. Centralbl. f. Bakt. (II) 14: 556. 1905.

² Kostytschew, S. und P. Eliasberg. Zeitschr. f. physiol. Chem. 118: 233. 1921.

³ Fitz. Ber. chem. Ges. 8: 1540. 1875; Pasteur, L. Etudes sur la bière, p. 126. 1876; Brefeld, O. Landwirt. Jahrb. 5: 281. 1876; Gayon. Mém. soc. sci. Bordeaux (2) 2: 249. 1878; Gayon et Dubourg. Ann. Inst. Pasteur. 1: 532. 1887; Hansen, E. C. Medd. frå Carlsberg lab. 2: 143. 1888; 5: 68. 1902; Calmette. Ann. Inst. Pasteur. 6: 605. 1892; Sanguinetti. Ibid. 11: 264. 1897; Henneberg. Zeitschr. f. Spiritusind. 25: 205. 1902; Sitnikoff. Ibid. 23: 391. 1900; Saito. Centralbl. f. Bakt. (II) 13: 158. 1904; Wehmer, C. loc. cit.

⁴ Kostytschew, S. und P. Eliasberg. loc. cit.

⁵ Calmette. loc. cit.; Sanguinetti. loc. cit.; and others.

⁶ Went und Prinsen-Geerligs. Verh. Akad. Wet Amsterdam. 1895; Collette et Boidin. Bull. Assoc. Sucr. et Distill. 16: 862. 1896; Henneberg. loc. cit.; and others.

ferments cane sugar and maltose¹ and even dextrins.² This fungus grows best at the extraordinarily high temperature of 40° and produces alcohol to the amount of 6.7%. Another fermentation fungus with a peculiar behavior is *Oidium lactis*, which ferments milk sugar much better than cane sugar, but which forms only a little alcohol. For other interesting fungi of fermentation see Lafar's "Handbuch der technischen Mykologie."

15. ANALYTICAL METHODS FOR THE DETERMINATION OF ALCOHOLIC FERMENTATION

The determination of the carbon dioxide developed by fermentation can be done by methods given above for the determination of plant respiration. In many cases simpler methods can be used because the rate of fermentation is considerably greater than that of oxygen respiration. For example, the determination of CO₂ by Meissl's method³ may be recommended. To a conical fermentation flask is added the Meissl trap (Fig. 42), the principle of which is that the gases which escape from the flask pass through concentrated sulphuric acid and are thus robbed of their water vapor. A rubber tube with a fine slit [sometime known as a Bunsen valve] is placed above and closed with a glass rod so that the gases can escape only through the slit. This prevents the absorption of water vapor from the surrounding air. Before an experiment is started, the flask is weighed with an accuracy of 0.01 g. A second weighing after the experiment makes it possible to measure the amount of carbon dioxide released. Of course, the determinations are more accurate if the air is entirely driven from the flask by CO₂ before the first weighing.

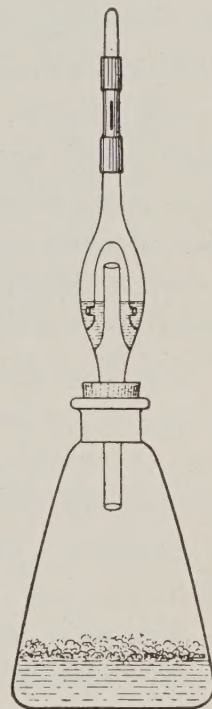


FIG. 42.—Fermentation flask with the Meissl trap. Conc. sulphuric acid is placed in the expanded part of the tube. The tube is closed above with a rubber valve.

Another method is to measure the increase in pressure in a fermentation flask connected with a mercury manometer.⁴ For rapid determinations, such as those required for investigations of the kinetics of an enzymatic process, it is desirable to follow the course of the fermentation, as Euler does, in an Ostwald thermostat and with constant shaking. The CO₂ is caught and measured in calibrated gas burettes over mercury. For preliminary estimates, it is advantageous to use a small bent graduated tube, into which a side piece is fused at the upper end while the other end remains open.⁵ A small amount of a mixture of yeast and sugar is added to the tube and the gas is measured in the closed side arm.

¹ Fischer, E. und P. Lindner. Ber. chem. Ges. 28: 3037. 1895; Buchner, E. und J. Meisenheimer. Zeitschr. f. physiol. Chem. 40: 167. 1903.

² Bau, A. Wochenschr. f. Brauerei. 9: 1185. 1892.

³ "Zymasegärung," p. 80. 1903.

⁴ Slaton, A. Jour. Chem. Soc. (London) 89: 128. 1906; Iwanoff, L. Centralbl. f. Bakt. (II) 24: 429. 1909.

⁵ Tollens, B. Handbuch d. biochem. Arbeitsmethoden by Abderhalden. 2: 142. 1910.

Determinations of alcohol can be made only in clear distillates. The fermented material is distilled until about half the total volume has passed over into the receiver. If the alcohol content does not exceed 5% it is sufficient to obtain a distillate of only a third or even a quarter of the total volume. If the first distillate is acid, it is rectified by the addition of calcium carbonate. Then one proceeds to qualitative and quantitative determinations of the alcohol.

The formation of iodoform is often used as a qualitative test for alcohol. The addition of fine crystals of iodine to an alkaline solution is followed by the formation of hexagonal crystals of iodoform, the characteristic form of which is easily recognized under the microscope.¹ This test is sensitive but not very reliable because iodoform is formed under the foregoing conditions not only from ethyl alcohol but also from acetaldehyde, acetone, lactic acid and a few other substances. Hence the formation of the ethyl ester of benzoic acid² is more reliable. The distillate to be tested is shaken with two drops of benzoyl chloride and an excess of potassium hydroxide. The characteristic odor of the ethyl ester of benzoic acid $\text{C}_6\text{H}_5\text{—CO—O—C}_2\text{H}_5$ develops in the presence of ethyl alcohol. Another sensitive test is to change the ethyl alcohol to acetaldehyde by careful oxidation; the latter gives many characteristic tests.³ The appearance of oily streaks in the upper part of the condenser at the beginning of the distillation indicates alcohol. After some practice it is possible to recognize the presence of ethyl alcohol with certainty by this method.⁴ However, the most sensitive methods for identifying ethyl alcohol are to change it either to the ethyl ester of para-nitro-benzoic acid $\text{C}_6\text{H}_4(\text{NO}_2)\text{CO—O—C}_2\text{H}_5$ ⁵ or to ethylene $\text{CH}_2=\text{CH}_2$.⁶ The resulting products are analyzed by the usual methods.

Alcohol is determined quantitatively by either physical or chemical methods. In large operations it is customary to make an areometric determination of the specific gravity of the solution of alcohol; an accurate pycnometer is used for delicate determinations. The alcohol content is calculated from the specific gravity by the help of special tables. It is perhaps still easier to use the cryoscopic method; the alcohol content is measured from the freezing point depression of the solution of alcohol, this being measured in the Beckmann apparatus.⁷ The stalagmometric method may be desirable. It is based on the lowering of the surface tension of water by the alcohol. This method has not yet been fully worked out.

The chemical methods of determining alcohol are preferable if very small quantities of alcohol are to be measured. The oxidation of the alcohol with

¹ Lieben. *Ber. chem. Ges.* **2**: 549. 1869. The most sensitive method was devised by Muntz. *Cf. Muntz, A.* *Ann. de chim. et de physique* (5) **13**: 543. 1878.

² Berthelot, M. *Compt. rend.* **73**: 496. 1871.

³ Denigés, G. *Bull. Soc. Chim.* (4) **7**: 951. 1910.

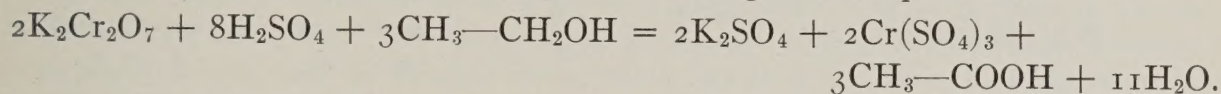
⁴ Pasteur, L. *Compt. rend.* **52**: 1260. 1861; Hansen, A. *Medd. frå Carlsberg lab.* **1**: 175. 1881.

⁵ Buchner, E. und J. Meisenheimer. *Ber. chem. Ges.* **38**: 624. 1905.

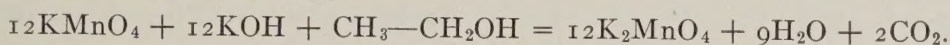
⁶ Berthelot, M. *Compt. rend.* **128**: 1366. 1899.

⁷ Gaunt, R. *Zeitschr. f. analyt. Chem.* **44**: 106. 1905.

bichromate and sulphuric acid according to Nicloux¹ is to be recommended particularly. 5 cc. of the distillate to be studied is placed in a test tube of Jena glass, and then about 6 cc. of concentrated sulphuric acid is added; the solution is then titrated at the boiling point with a suitable solution of potassium bichromate until the blue-green color changes to yellow-green. The alcohol content is easily calculated from the amount of bichromate solution used. The oxidation of alcohol to acetic acid goes according to the equation:

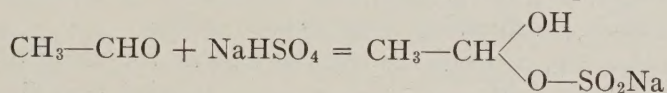


Another method is to burn the alcohol to CO_2 and H_2O by potassium permanganate in alkaline solution at the simmering point:



Further information concerning this method can be found in the original paper by Barendrecht.²

For quantitative determinations of alcohol, the distillate may contain no more than 0.01% of acetaldehyde or errors will appear because acetaldehyde is affected by the foregoing oxidation methods in the same way as ethyl alcohol. The acetaldehyde may be removed in two ways. According to the first method,³ a concentrated solution of sodium bisulphite is added to the distillate and the mixture is distilled on the water bath at 30–35° and with about a 15 mm. pressure. The condenser is cooled with ice. The alcohol passes into the distillate while the acetaldehyde remains behind in the form of a bisulphite compound:



The distillate is then made slightly alkaline because a small amount of sulphurous acid always passes over into the receiver.

Another method of removing the acetaldehyde is to fix it with meta-phenylene-diamine. The resultant compound is not volatile and therefore does not pass into the receiver.⁴

16. INDUSTRIAL APPLICATIONS OF ALCOHOLIC FERMENTATION

Until recently, alcoholic fermentation by yeast was the only industrial method of producing alcohol. Fermentation is also used in the manufacture of alcoholic drinks (wine, beer, cider, kefir and koumiss) and of the pressed yeast used by bakeries. For all these important fields of industrial chemistry there is an abundance of technical literature; hence it is quite impossible to go into the details of these industrial processes. However, the behavior of the microorganisms in the fermentation industries is important in some respects for theoretical plant physiology; we shall now give our attention to these subjects.

The manufacture of wine is by far the oldest fermentation industry. It consists in pressing the sweet must containing grape sugar⁵ from the grapes and subjecting it to alcoholic fermentation. If the must is relatively poor in sugar, the latter will be totally fermented and the result will be the so-called dry wine, but if the sugar content is so high that fermentation is stopped by the high content of alcohol before all the sugar is fermented, the result is a sweet wine containing sugar. Very strong wines (with an alcohol content of 16% or more) are prepared by adding alcohol after the sugar is all fermented. Frothy wines are allowed to ferment for awhile in

¹ Nicloux, M. Bull. Soc. Chim. 35: 330. 1906.

² Barendrecht, H. P. Zeitschr. f. analyt. Chem. 52: 167. 1913; see also Kostytschew, S. Mitt. d. Akad. Wiss. Petersburg, p. 327. 1915.

³ Kostytschew, S. loc. cit.; also Biochem. Zeitschr. 64: 237. 1914.

⁴ Neuberg, C. und J. Hirsch. Biochem. Zeitschr. 98: 111. 1919.

⁵ The must of grapes contains no trace of cane sugar. If cane sugar is found in wine it was added when the wine was made.

corked bottles and the wine is accordingly super-saturated with CO_2 . The flavor or the so-called bouquet of wines depends on various ester-like substances, the nature of which is not yet understood. These substances are formed in part by the fermentation itself: in addition, they accumulate as the grapes ripen and during the storage of the wine in cellars. The best wines from Krymsk contain, according to special analyses, the following quantities of grape sugar, alcohol, volatile and non-volatile acids:

Brand of wine	Alcohol in %	Grape sugar, %	Volatile acids (acetic acid, etc.), %,	Non-volatile acids (tartaric acid, etc.), %
Table wine, white.....	9.05	0.11	0.15	0.33
Table wine, red.....	7.85	0.14	0.14	0.43
Cabernet, red.....	10.11	0.26	0.13	0.50
Pineau-Chardonnnet, white.....	11.40	0.19	0.12	0.34
Muscatel, red.....	8.47	26.71	0.11	0.45
Muscatel, white.....	7.06	37.28	0.06	0.56
Cabernet-port wine.....	14.74	3.99	0.07	0.54
Port wine, red.....	16.50	10.93	0.09	0.43
Port wine, white.....	13.78	12.82	0.16	0.49
Pineaux gris-port wine.....	14.02	13.67	0.12	0.42

This table includes wines with a particularly low content of volatile acids. Caucasian wines, for example, always contain considerably more volatile acids.

Previous to the publication of Pasteur's epochal papers, the idea prevailed that the grape juice ferments spontaneously. His exact experiments showed that there is always a large amount of yeasts on all grapes, especially on the stems. Wine yeasts form the species *Saccharomyces ellipsoideus*. Even to-day wines are usually made without the addition of yeast because the quality of the wine depends chiefly on the culture of the grape and the way the wine cellar is kept.

The brewing business is just as great an industry as growing grapes. In general, beer is made as follows. The material used is germinating seeds of barley or wheat. At a certain stage of germination, when the amount of amylase in the seeds reaches a maximum, the seeds are killed by careful drying and the so-called malt is thus obtained. This is allowed to stand undisturbed in water at the temperature of $50-70^\circ$ which is optimum for amylase. The starch is thereby hydrolyzed to maltose and the result is a deep-brown, sweet solution, the so-called wort. The wort is boiled with hops to impart a good flavor to the beer, and then cooled quickly and yeast is added. The yeast ferments the sugars (chiefly maltose) in the wort to alcohol and CO_2 . The strains of yeast do not all ferment in the same way. There are two principal types of fermentation—top and bottom fermentation. The first takes place violently at room temperature and is finished in 2–3 days; the wort foams and large masses of yeast rise with the froth and float at the surface in the form of slimy islands. The temperature reaches 21° and a greater increase in temperature is unfavorable and is prevented by cooling. Bottom fermentation is caused by other strains of yeast. An increase in temperature of more than 5 or 6° is prevented by cooling with ice. The yeast remains at the bottom of the container, the sugar is fermented slowly and usually incompletely. Beer prepared in this way must therefore be kept constantly at a lower temperature or the product may spoil. This difficulty of handling it is compensated by the superior flavor of the beer fermented from the bottom. It has replaced the beer fermented by top yeasts in all countries except England.

For many brands of beer, the so-called secondary fermentation is very important. The secondary fermentation takes place in the corked bottles and is weak in comparison with the main fermentation. In the making of beer, the taste and the bouquet of the products depends

to a large extent on the kind and manner of fermentation. It is therefore understandable that in growing grapes, most of the attention is given to the varieties of grapes, the nutrition and fertilization of the grapes and the conditions in the wine cellar while in the brewing business the strains of yeast are the most important.

During the secondary fermentation, the beer receives a good flavor, becomes clear and saturated with CO₂; a beer without CO₂ is impalatable. Secondary fermentation is especially important for the beers fermented from the top. The secondary fermentation uses the dextrans which remain practically intact during the main fermentation.

We now recognize several races of beer yeasts which differ from each other by various biochemical characteristics. The description of all these races, their isolation in pure culture, as well as the general methods of isolation and pure culturing of the different yeasts we owe to Hansen. He also worked energetically to have the large brewing industry rely on pure cultures of yeasts. The use of pure cultures alone guarantees a product of constant properties and prevents spoiling of the beer. Damage to the product consists largely in that the beer becomes bitter as the result of the development of wild forms of yeast which, of course, occur only in impure yeast cultures. Hansen proposed the following simple method for detecting wild strains of yeast. On plates of gypsum and because of the lack of water, all strains of yeast form ascospores which are easily recognized under the microscope. At room temperature (about 15°) wild yeasts give mature ascospores after three days while no cultivated strains can form ascospores in such a short time.

Hansen's method of pure cultures in large operations soon gained adherents in Denmark and various German industries but it met with stubborn resistance in England. It was argued there that the properties of some of the famous beers are due to the activity of a mixture of different races of yeast in that, for example, there is one strain of yeast for the primary fermentation and another for the secondary fermentation. The British investigators Brown and Morris, who directed a large brewery for a long time, were urged, after some years of tests, to give up pure cultures, because they did not make it possible to prepare a beer with the long-known "normal" properties. Microbiological tests also showed that the composition of the mixtures of yeasts used for the old famous brands of beer, remains surprisingly constant. This is a partnership between strains of yeast adapted to each other for many years. Brown and Morris have carried out interesting experiments in this field, of which the following may be mentioned. The primary fermentation was started by the amount of yeast which is used to make the Burton ale. The alcohol was distilled off and the wort again inoculated with the same amount of yeast. The strain which then developed exclusively was that which produces the secondary fermentation; the principal strain *Saccharomyces cerevisiae* showed no growth. In this way it was found that the primary fermentation and the secondary fermentation are produced by different races of yeast. On the basis of these facts, a few experts claim that pure cultures of yeast cannot be used under certain conditions. The followers of Hansen's views contradict this statement and the debated question is not yet conclusively solved. Since there is no large amount of sugar in the beer wort and fermentation proceeds slowly in most cases, the alcoholic content of beer cannot be very high. The following table gives the content of some beers as to sugar and products of fermentation.

	Alcohol, %	CO ₂ , %	Sugar, %
Summer beer.....	3.21	0.23	0.44
Porter.....	5.35	0.22	1.34
Pale ale (wheat beer).....	2.51	0.28	0

The preparation of alcohol and strong alcoholic drinks is analogous in principle to the making of beer but the liquid is enriched with carbohydrates. The characteristics of the different strains of yeast are not very important in this operation. One must use yeasts which are not sensitive to high concentrations of alcohol. The material for the alcohol industry is cereal

grain (rye) and potatoes. The latter is preferable because 1 hectare of potatoes yields almost three times as much starch as 1 hectare of rye. For drinking purposes, however, the alcohol from potatoes must be carefully purified because it contains so much fusel oil.

The materials are divided into small pieces and boiled with water at a high pressure. By suddenly opening the tap of the boiler there is a violent formation of the steam and this breaks the cell walls of the material. The subsequent change of starch to sugar then proceeds more rapidly. Malt is then added to the macerated hot mass and the mixture is handled as in the manufacture of beer. Not only the starch of the malt but also that of the macerated material is changed to sugar. The sugar is then fermented by top yeast and the result distilled. The resultant raw alcohol usually has a strength of about 80% and is rich in fusel oils. The alcohol is purified in special rectifying apparatus and at the same time the content of alcohol is increased to 96%. This is the highest content of alcohol possible by simple distillation without chemical treatment.

The pressed top yeast required by the baking industry is a useful by-product of the alcohol industry. About 20% potato starch is usually added to the yeast to make it a compact mass and the mixture is pressed into tablets. In recent years, good pressed yeast has been prepared without adding starch. As the market requires, the pressed yeast under certain conditions becomes the principal product and alcohol a by-product of the alcohol industry. In the latter case a strong current of air is passed through the fermenting mass because this makes a considerable increase in the amount of yeast. Strong alcoholic drinks are made from alcohol to which various substances are added. Rum and arak are made from alcohol from which the fusel oil has not been removed; likewise cognac comes from the alcohol of wine. In rum the alcoholic content reaches 77.5%.

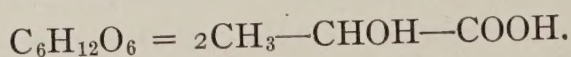
Fruit wines are prepared in the same way as grape wines but cane sugar is added to the juice because most fruit is poor in sugar. Usually no yeast is added because various yeasts always settle spontaneously on the fleshy fruits.

Kefir and koumiss are products of the alcoholic fermentation of milk which is produced by various specific microorganisms. These are able to hydrolyze milk sugar. At the same time lactic acid fermentation also takes place in the milk. Alcoholic sour beverages of outstanding dietetic importance are formed as the result of these simultaneous fermentations. Both kefir and koumiss were first used by Asiatic peoples. The preparation of kefir requires cow's milk which is fermented by *Torula kephir*.¹ Its alcoholic content only reaches 0.7–0.8%. Koumiss is prepared from mare's milk by fermentation with *Torula Kumys*.¹ In koumiss the alcoholic content rises to 2% and more.

17. LACTIC ACID FERMENTATION

It has already been mentioned that one molecule of sugar is broken up into two molecules of lactic acid during lactic acid fermentation. Milk is an especially favorable substrate for lactic acid fermentation.

Pasteur² was the first to prove that lactic acid fermentation is a biological process. Milk sugar is first hydrolyzed by specific bacteria to form a mixture of equal quantities of glucose and galactose; then the hexoses are broken into lactic acid. The bacteria of lactic acid fermentation are divided into two groups. *Bacterium lactis acidi* Leichm. (Fig. 43) are typical of the first group. This organism is entirely independent of atmospheric oxygen. It ferments all the milk sugar, even in deep layers of milk, to lactic acid.³ The equation for this kind of lactic acid fermentation is thus very simple:



¹ Duclaux, E. Ann. Inst. Pasteur. 1: 573. 1887; 3: 201. 1889.

² Pasteur, L. Compt. rend. 45: 913. 1857; 47: 224. 1858; 48: 337. 1859.

³ Leichmann. Milchzeit. 25: 67. 1896; Kayser, E. Ann. Inst. Pasteur. 8: 737. 1894.

Direct analyses actually show that the amount of lactic acid formed by this type of lactic acid fermentation amounts to at least 98% of the theoretical yield. Another form very common in milk is *Bact. acidi lactici* Hueppe but it forms not only lactic acid but also considerable quantities of ethyl alcohol and acetic acid.¹ This is a representative of the second kind of producer of lactic acid fermentation, which develops preferably with access to oxygen, ferments the upper layers of milk and thereby produces other products of fermentation besides lactic acid. The extreme type is *Bact. lactis aerogenes* which forms a large amount of various gases, especially CO₂, hydrogen (52%) and methane, and also produces acetic acid, lactic acid and ethyl alcohol. The output of acetic acid in this fermentation is often greater than that of lactic acid; according to this it would be fair to speak of this case as an acetic acid fermentation of sugar.² Still there are various chemical reasons to be explained later for thinking that there are only three types of true fermentations—alcoholic, lactic acid and butyric acid fermentation.

If we review the discussion of lactic acid fermentation, we conclude that it is divided into pure and impure lactic acid fermentation. In the first case the sugar is split in accordance with the theoretical equation of lactic acid fermentation, but in the second case products other than lactic acid are formed in such amounts that lactic acid is often left in the background.

Several types of producers of lactic acid fermentation have been isolated and studied in pure cultures. One produces the optically inactive dl-lactic acid, another forms dextro-lactic acid and a third levo-lactic acid. Some aerobic strains consume the acid formed; the chemical side of this use of lactic acid is not yet made clear. It is also undecided how the different optically isomeric lactic acids arise. In this field one hits upon mutually contradictory facts. It is remarkable that the same species of bacteria, for example the common *Bact. lactis acidi*, produces inactive lactic acid when grown on cow's milk but forms dextro-lactic acid in pure cultures on artificial nutrient media.³ Other bacteria form only levo-lactic acid.⁴ The formation of optically inactive lactic acid has been explained as the production of the two optical isomers by different bacteria at the same time but this explanation proved to be untenable.

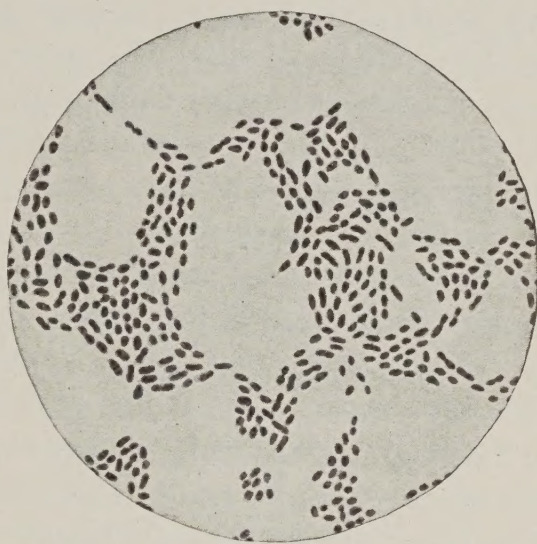


FIG. 43.—*Bacterium lactis acidi* Leichm. Magnified 1000 X. (After Omeliansky.)

¹ Haacke, P. Arch. f. Hyg. 42: 16. 1902.

² Baginski, A. Zeitschr. f. physiol. Chem. 12: 434. 1888.

³ Günther, C. und Thierfelder. Arch. f. Hyg. 25: 164. 1895; Hyg. Rundschau. 10: 769. 1900; Kozai. Zeitschr. f. Hyg. 31: 337. 1899; 38: 386. 1901; Leichmann. Milchzeit. 25: 67. 1896; Péré. Ann. Inst. Pasteur. 7: 737. 1893; 12: 63. 1898; Pottevin. Ibid. 21: 49. 1898; Utz. Centralbl. f. Bakt. (II) 11: 600. 1904; and others.

⁴ Schardinger. Monatsch. f. Chem. 11: 545. 1900; Leichmann. loc. cit.; Kozai. loc. cit.; Utz. loc. cit.; Leichmann. Centralbl. f. Bakt. (II) 5: 344. 1899; and others.

Perhaps this question might be of general interest in that the appropriate investigations might contribute to the explanation of the mechanism of the formation of optically active substances in living cells, but as yet there has been no conscious, systematic investigation of this matter.

The lactic acid bacteria are exacting in respect to their nitrogenous nutrition. A good growth is obtained only in the presence of peptone or soluble proteins.¹ The bacteria of the aerogenes type develop on single amino acids or even on ammonium salts² but they are entirely unable to use nitrates. Lactic acid bacteria do not always grow well on solid nutrients. The best growth is found in milk yet they do well on artificial media containing sugar and peptone. All lactic acid bacteria ferment glucose, fructose, mannose, galactose and maltose. Most of them also ferment milk sugar but only a few attack cane sugar.³ The important form *Bact. lactis acidi* Leichm. contains no invertase. The optimum temperature is usually not less than 30°; at times it reaches 40°. Unlike alcoholic fermentation, lactic acid fermentation is usually impossible at a temperature below 12°.

Just as alcoholic fermentation is brought to a standstill by the accumulation of alcohol, lactic acid fermentation is possible only if the acid content remains below a certain point. The limiting concentration is not the same for the different bacteria but is low as a rule. Thus *Bact. acidi lactici* Hueppe can tolerate only 0.58–0.8% of free acid; for *Bact. lactis acidi* Leichm. the limit of concentration is 0.54–1.25%, etc.⁴ However, if the lactic acid fermentation proceeds in the presence of calcium carbonate, all the sugar is always fermented because there is no chance for an acid reaction in this case.

Some investigators point out that many lactic acid bacteria are not entirely indifferent to the atmospheric oxygen, because the fermentation always goes better with oxygen excluded.⁵ According to special experiments, a good growth of *Bact. lactis acidi* Leichm. and of *Bact. caucasicum* is obtained on solid nutrients with oxygen entirely excluded while this was not possible with oxygen present. With restricted access to oxygen, *Bact. lactis acidi* Leichm. gives off no gases⁶ and is therefore entirely engaged in fermentation. According to the latest special experiments, *Bact. caucasicum* or *Bact. lactis acidi* Leichm., even with perfect aeration, has at most only a very slight oxygen respiration.⁷ This is a sharp distinction from the behavior of yeasts; the latter get working energy with oxygen present by means of oxygen respiration (p. 407 ff.) while the producers of pure lactic acid fermentation live at the expense of the process of fermentation both with and without access to oxygen.

¹ Kayser, E. Ann. Inst. Pasteur. 8: 737. 1894; Marshall, C. E. Centralbl. f. Bakt. (II) 11: 739. 1904; Jensen. Ibid. 4: 196. 1898.

² Hueppe. Mitt. d. Reichs-Gesundheitsamtes. 2: 309. 1884; Fraenkel, C. Hyg. Rundschau. 4: 769. 1894; Kozai. loc. cit.

³ Cf. Weigmann. Handb. d. tech. Mykologie by Lafar. 2: 92. 1905.

⁴ Hueppe, F. loc. cit.; Aderhold. Centralbl. f. Bakt. (II) 5: 511. 1899; and others.

⁵ Kayser, E. loc. cit.; McDonnell. Diss. Kiel. 1899; Troili-Peterson. Zeitschr. f. Hyg. 32: 361. 1899; Henneberg, W. Zeitschr. f. Spiritusind. 26: 226. 1903; and others.

⁶ Euler, H. v. und O. Svanberg. Zeitschr. f. physiol. Chem. 102: 176. 1918.

⁷ Kostytschew, S. und M. Afanassjewa. Compt. rend. July, 1925.

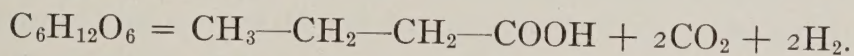
Lactic acid fermentation is used in the dairy industry to prepare sour milk and dairy products. (The ripening of cheese is a complicated process in which, among others, the butyric acid bacteria also take part.) The lactic acid bacteria also play an important rôle in the making of sauerkraut, sour dough, etc.

18. BUTYRIC ACID FERMENTATION

This third and last kind of true fermentation was also discovered by Pasteur.¹ He was also the first to prove that the agents of butyric acid fermentation are obligate anaerobes; in other words, they are living forms which can develop normally only in the complete absence of oxygen.² Thus there are the following types of microorganisms to be distinguished: 1. Aerobic forms which can grow only in the presence of oxygen,³ 2. Facultative anaerobic forms which can live either with or without oxygen, and 3. Obligate anaerobic forms which develop only with oxygen excluded.⁴

There are different species of butyric acid bacteria. Prazmowski⁵ set up the genus *Clostridium* and under the name *Clostridium butyricum* apparently described the form which Pasteur first isolated in his discovery of butyric acid fermentation. In Chap. III was described the likewise obligate anaerobic species *Clostridium Pasteurianum* which produces butyric acid fermentation and fixes molecular nitrogen. According to some authors, the capacity to fix nitrogen to some extent is peculiar to all species of the genus *Clostridium*. In recent years the species *Clostridium butyricum* is divided into three species called *Clostridium butyricum* I, II and III.⁶ Some investigators collect various species of *Clostridium* under the name *Granulobacter*;⁷ on the whole, the classification of the butyric acid bacteria is somewhat involved.

The non-motile *Bac. butyricus* Botk.,⁸ a strongly anaerobic form, is very common. Bacteria have also been described which can live in the presence of oxygen.⁹ These include some pathogenic microorganisms. The theoretical equation for butyric acid fermentation is:



Each molecule of sugar is broken into 1 molecule of butyric acid, 2 molecules of carbon dioxide and 2 molecules of hydrogen. Various by-products are often

¹ Pasteur, L. Compt. rend. 52: 344 and 1260. 1861; 56: 416 and 1189. 1863.

² Chudiakow, N. Centralbl. f. Bakt. (II) 4: 389. 1898; The theory of anaerobiosis. 1896 (Russian) showed that butyric acid fermentation is actually stopped by the entrance of oxygen because the obligate anaerobic promoters of fermentation die.

³ In this book a distinction is also made between "strongly aerobic" and "aerobic but fermenting" microorganisms. The latter can live without oxygen for a time in the growing condition, while strongly aerobic forms die quickly without oxygen even in the growing condition.

⁴ The difference between facultative and obligate anaerobes is really only quantitative because there are scarcely any organisms which are entirely indifferent to oxygen.

⁵ Prazmowski, A. Untersuchungen über Entwicklung und Ferment-wirkung einiger Bakterienarten. 1880; Paschutin. Pflügers Arch. f. ges. Physiol. 8: 352. 1874.

⁶ Gruber, M. Centralbl. f. Bakt. 1: 367. 1887.

⁷ Beijerinck. Über die Butylalkoholgärung und das Butylferment. 1893; Bredemann. Ber. bot. Ges. 26a: 362. 1908; Idem. Centralbl. f. Bakt. (II) 23: 1. 1909; Kemp. Ibid. (I) 48: 54. 1908; Kirow. Ibid. (II) 3: 535. 1911.

⁸ Botkin, S. Zeitschr. f. Hyg. 11: 421. 1892.

⁹ Hueppe. Mitt. d. Kais-Gesundheitsamtes. 2: 353. 1884; Loeffler. Berlin. Klin. Wochenschr. Vol. 24, no 33. 1887; and others.

formed in still larger amounts than in lactic acid fermentation. It is evident from the foregoing explanations that some bacteria produce pure lactic acid fermentation but so far not a single promoter of butyric acid fermentation has been isolated which effects a cleavage of sugar from the start to the finish of fermentation exactly according to the theoretical equation. The usual by-products of butyric acid fermentation are ethyl alcohol, acetic acid and lactic acid. The latter is very often formed during butyric acid fermentation. Hence, it is not always easy to tell whether a given fermentation is to be called lactic acid fermentation or butyric acid fermentation, if the various by-products are being formed during the cleavage of sugar. Often a certain by-product, such as acetic acid, is stored up only at the beginning of the process of butyric acid fermentation. As a rule, butyric acid fermentation is a less regulated process than alcoholic or even lactic acid fermentation. On the contrary, butyric acid bacteria are unassuming in respect to the material for fermentation: they ferment not only sugars but also starch, lactic acid and in some cases even glycerine.¹ Butyric acid fermentation is generally common in nature. It takes place in every mud free from oxygen and it is never lacking in moor soils. In the process of butyric acid fermentation, most of the organic substance is split under anaerobic conditions.

A most interesting variety of butyric acid fermentation is the fermentation of cellulose which is produced by specific bacteria. Cellulose is a particularly stable form of polysaccharide; it is not attacked by cold acids and alkalies and in general it is extremely hard for bacteria to split it. Therefore the contents of the dead plant cells is quickly taken by soil bacteria but the cell walls remain intact longer, as, for example, in the case of fallen leaves. In the presence of oxygen, a few molds and bacteria are able to oxidize cellulose² but when oxygen is excluded this substance is used exclusively by the peculiar process of fermentation. Pure cultures of cellulose bacteria were first isolated by Omeliansky³ by the use of the elective culture method of Winogradsky (p. 149). In these experiments the carbon food was filter paper. It was found that there are two kinds of cellulose fermentation—methane fermentation and hydrogen fermentation. In the first, CO₂, methane, much acetic acid and butyric acid are formed. The amount of the gases is approximately equal to that of the acids. Hydrogen fermentation occurs if the first inoculation culture is warmed to 75°. In this case the amount of gas is small. The gas consists of CO₂ and hydrogen. Small quantities of formic acid and valeric acid are formed in addition to acetic and butyric acid. The exciter of methane fermentation was named *Bacillus cellulosae methanicus* and that of hydrogen is called *Bacillus cellulosae hydrogenicus*. Both are thin rods which form the spore at one end and thereby take the form of drum-sticks (Fig. 44).

¹ Iterson, C. v. *Centralbl. f. Bakt.* 11: 689. 1904; Pringsheim, H. *Zeitschr. f. physiol. Chem.* 78: 282. 1912; *Centralbl. f. Bakt.* 23: 300. 1909; 26: 222. 1910; Christensen. *Ibid.* 27: 449. 1910; Kroulik. *Ibid.* 36: 339. 1912; and others.

² Winogradsky, S. *Compt. rend. acad. sci. Paris.* 183: 691. 1926; 187: 326. 1928.

³ Omeliansky, W. *Compt. rend.* 121: 653. 1895; 125: 970 and 1131. 1897; *Idem.* *Centralbl. f. Bakt. (II)* 8: 193. 1902; 11: 370 and 703. 1903; 15: 673. 1906; 36: 339. 1912; *Idem.* *Arch. des sci. biol.* 7: 411; Vol. 9. 1900; Vol. 12. 1905.

Pectins are broken down under the influence of specific bacteria. This process is done either by oxidation or by a variety of butyric acid fermentation. One promoter of pectin fermentation goes by the name *Granulobacter pectinovorum*.¹ The so-called steeping of flax consists in the maceration of the bast fibers under the influence of pectin fermentation. Flax can be steeped either aerobically or anaerobically. In the first case, the flax is spread out on the ground and the intra-cellular substance is slowly destroyed by various aerobic bacteria. In the second case, the flax is immersed in standing water where pectin fermentation sets in. The idea has become widespread in recent times that flax-steeping should be carried out by means of pure cultures of pectin fermentation bacteria; otherwise part of the bast fibers of the flax can always be decomposed by cellulose fermentation, since the two fermentations so often proceed simultaneously in nature.

The chemical side of pectin fermentation is very interesting; considerable quantities of butyl alcohol and acetone are thereby formed.

As early as the year 1912, Fernbach and Strange² patented a biological method of preparing acetone and butyl alcohol. This method is now being used to a large extent. For example, two large plants in United States are producing 60 tons of butyl alcohol and 20 tons of acetone daily. Various bacteria but chiefly those of pectin fermentation are being used for this. They require the complete exclusion of air and attack directly the unhydrolyzed starches.³ A third of the starch is converted to butyl alcohol (60%), acetone (30%) and ethyl alcohol (10%). In addition, considerable quantities of CO₂ and hydrogen are formed. However, if CaCO₃ is added to the fermenting mixture the result is ordinary butyric acid fermentation. This shows that butyl alcohol fermentation is a variety of butyric acid fermentation.⁴ Eliasberg⁵ also obtained the reverse transformation; in the case of the fermentation of *Clostridium Pasteurianum* (p. 169)—a typical agent of butyric acid fermentation—considerable amounts of butyl alcohol are formed if the fermentation goes on in an atmosphere of hydrogen under high pressure (80–100 atmospheres). In young cultures, more than half the butyric acid is replaced by butyl alcohol. Butyl alcohol is also obtained with a high tension of nitrogen but in much smaller amounts.

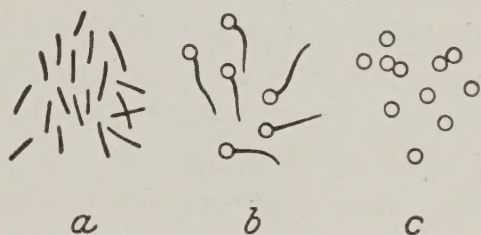


FIG. 44.—Bacteria of cellulose fermentation. (a) young cells (b) "drum-sticks" (c) ripe spores. Magnified 1000 X. (After Omeliansky.)

¹ Winogradsky, S. and Friebe. Compt. rend. 121: 742. 1895; Behrens, J. Centralbl. f. Bakt. (II) Vol. 8. 1912; 10: 524. 1903; Beijerinck and van Delden. Arch. Néerland. de Physiol. de l'homme et des anim. (2) 9: 418. 1905; Schardinger. Centralbl. f. Bakt. (II) 19: 161. 1907.

² Fernbach and Stange. Eng. Pat. No. 21073. 1912; German Pat. No. 323533. 1912.

³ Gill, A. Jour. Soc. Chem. Ind. 38: 2731. 1919; Speakman, H. B. Ibid. 38: 1154. 1919; Jour. Biol. Chem. 41: 319. 1920; 58: 395. 1923; Thaysen, A. C. Chem. and Ind. 45: 71. 1926; Killetter. Ind. and Eng. Chem. Vol. 19. No. 1. 1927.

⁴ Reilly, I., Hickinbottom, Henley and Thaysen. Biochem. Jour. 14: 229. 1920; Wilson, Peterson and Fred. Jour. Biol. Chem. 74: 495. 1927.

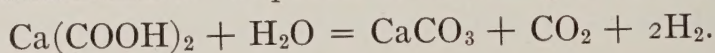
⁵ Eliasberg, P. An unpublished paper.

Another interesting group of bacteria which is collectively termed *Bact. macerans*, produces acetone and ethyl alcohol in which case butyl alcohol is not formed.¹

In both cases of acetone formation it is thought that acetone is formed from acetaldehyde by way of aldol, hydroxy-butyric acid and acetoacetic acid $\text{CH}_3\text{—CO—CH}_2\text{—COOH}$.

It is impossible to describe here the large number of bacteria which bring about various fermentations which are to be regarded as intermediate between lactic acid fermentation and butyric acid fermentation. It must be emphasized that different fermentations which at first glance seem to be original are actually varieties or combinations of the three types of fermentation described above. For example, in sugar factories there is often an astonishing development of *Leuconostoc mesenteroides* which changes enormous quantities of sugar to a tasteless slime composed of polysaccharides.² This process is often called slimy fermentation but in principle it is incorrect because the polymerization of sugar is not an exothermic process and can therefore not be a source of energy. In fact there is also a sudden start of a secondary process since *Leuconostoc* produces lactic acid fermentation of the aerogenes type with the formation of lactic acid, acetic acid, formic acid, ethyl alcohol and CO_2 . This fermentation furnishes the working energy for *Leuconostoc*. Various other lactic acid bacteria often form a slime of polysaccharides.³

The so-called formic acid fermentation likewise seems at first sight to be a special type of fermentation. It proceeds according to the equation:⁴



But the heat production of this peculiar reaction is 0 (it is really expressed by a small negative value). Under natural conditions the bacteria concerned produce impure lactic acid fermentation which forms lactic acid, acetic acid, formic acid, ethyl alcohol, CO_2 and hydrogen. The "formic acid bacteria" (*Bacterium formicicum*) carry out this fermentation at the expense of various kinds of sugars.

19. OXIDATIVE FERMENTATIONS

On the basis of the foregoing explanations, all true fermentations are to be traced to three principal types—alcoholic, lactic acid and butyric acid fermentation in addition to the intermediate stages. By true fermentations we only mean cleavage processes which can take place with oxygen entirely excluded. Quite different are the oxidative fermentations which occupy an intermediate position between true fermentations and oxygen respiration. They are oxidations which take place at the expense of atmospheric oxygen and in this sense

¹ Schardinger, F. Centralbl. f. Bakt. (II) 14: 772. 1905; Speakman, H. B. Jour. Biol. Chem. 64: 41. 1925.

² Cienkowski. Arb. d. Naturforscherges. zu Charkow. Vol. 12. 1878; van Tieghem. Ann. sci. nat. (6) 7: 180. 1878.

³ Panek, K. Bull. Acad. Cracovie, p. 5. 1905; Laxa, O. Centralbl. f. Bakt. (II) 8: 154. 1902; Smith and Steel. Ibid. 8: 596. 1902; Schardinger, F. Ibid. 8: 144. 1902; Emmerling, O. Ber. chem. Ges. 33: 2478. 1910; and others.

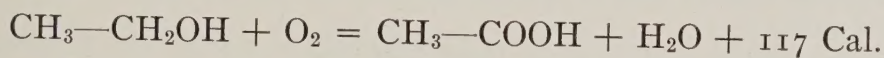
⁴ Omeliansky, W. Centralbl. f. Bakt. (II) 11: 177. 1903.

are analogous to oxygen respiration. However, there is not a complete combustion; the products of oxidative fermentations are always combustible organic substances. Consequently there is a considerable consumption of material by oxidative fermentations. In this respect they are analogous to true fermentations.

The best known and widely distributed oxidative fermentation is the commercially useful acetic acid fermentation. The material for this fermentation is ethyl alcohol which is oxidized to acetic acid by specific bacteria. This important process was also first correctly explained by Pasteur.¹ The producers of the fermentation are various species of bacteria. The first to be known were the very common species *Bacterium aceti* (Fig. 45) and *Bacterium Pasteurianum*.² Pasteur performed his classic investigations with these bacteria. Later the very common species *Bact. xylinum*³ and *Bact. Kützingerianum*⁴ were described. At present we know of other promoters of acetic acid fermentation, including even a mold.⁵ *Bact. orleanense* is the most powerful exciter of fermentation.⁶ The equation for acetic acid fermentation is very simple:



FIG. 45.—*Bacterium aceti* Hans. Magnified 1000 ×. (After Omeliansky.)



Unlike the true fermentations, this reaction can easily be carried out by purely chemical means. It furnishes only about a third as much energy as the combustion of alcohol to CO_2 and H_2O .

According to Pasteur's data, the rate of fermentation and of reproduction of the vinegar bacteria is very high. In a couple of days each cell can oxidize a quantity of alcohol equal to 10,000 times the weight of the agent of fermentation, if the limiting concentration of acetic acid which stops the fermentation is not reached. This limit is rather high in most cases. For *Bact. aceti* and *Bact. Kützingerianum* it is 6.6%, and for *Bact. Pasteurianum* it is 6.2%.⁷ Acid contents of 10% or 14% have been observed in some cases.⁸

In the case of true fermentations, the organisms occur at the bottom of the vessel when the fermenting solution is deep. On the contrary, the vinegar bacteria form a connected scum [known as "mother"] at the surface of the

¹ Pasteur, L. Compt. rend. 54: 265. 1862; Etudes sur la vinaigre. 1868.

² Hansen, E. C. Medd. frå Carlsberg lab. Vol. I. 1870.

³ Brown, A. J. Jour. Chem. Soc. (London) 49: 432. 1886.

⁴ Hansen, E. C. Medd. frå Carlsberg lab. 4: 265. 1894.

⁵ Lafar, F. Centralbl. f. Bakt. 13: 687. 1893.

⁶ Henneberg, W. Centralbl. f. Bakt. (II) 3: 223. 1897; Idem. Deutsch. Essigind. 10: 89. 1906.

⁷ Henneberg, W. loc. cit.

⁸ Steinmetz, O. Chem. Zeit., p. 1723. 1892; Bokorny, Th. Centralbl. f. Bakt. (II) 12: 484. 1904; Janke. Ibid. 45: 145 and 534. 1916; 46: 545. 1916.

solution. This membrane absorbs oxygen from the air and uses it to oxidize the alcohol. The fabulous rate of reproduction of the vinegar bacteria can best be illustrated by the fact that an inoculation of a suitable nutrient solution with the minimum amount of bacteria gives a scum of 1 m.² in 24 hours. Of course, this scum consists of an enormous number of individual bacteria.

The French or so-called Orléans method of making vinegar is based on the formation of such a bacterial scum. Broad, shaded, well-ventilated vats are filled with spoiled wine and allowed to stand undisturbed at 20–30°. On the surface of the liquid there is a rapid growth of a scum of vinegar bacteria which change the alcohol to acetic acid. The wine vinegar is considerably more esteemed than ordinary vinegar which is prepared by diluting acetic acid. The former contains various aromatic esters which are formed partly in the wine and partly during the fermentation and to these wine vinegar owes its peculiar flavor. The vinegar industry is an important adjunct to the making of wine. Unsuccessful or spoiled wine is converted into vinegar.

The Orleans vinegar industry is very old but it was placed on a scientific basis only by Pasteur's pioneer work. Previous to the proof of the biological nature of acetic acid fermentation, the formation of the scum depended on a spontaneous inoculation and it was all a matter of chance. Under certain conditions, for example, there was a production not of acetic acid but of acetaldehyde, a poisonous substance with a pungent odor. Now we know that this is produced by a pathological condition of the bacteria. The best means of doing away with this trouble is to remove the diseased film and reinoculate, all of which takes very little time. But since at that time it was impossible to form a film artificially, the destruction of the scum was avoided if possible. The result was a loss of raw material when the expensive film was kept in the hope that it would improve. For this reason it was impossible to regulate the production of vinegar. It was often necessary to make the most of the work of the film on a large stock or, on the contrary, it was a hard test of patience in case the film died out when market conditions were favorable.

After Pasteur's studies made clear the theoretical nature of acetic acid fermentation, a new epoch began in the vinegar industry. If a fresh film can be easily and with certainty obtained in 24 hours by inoculation, it is unnecessary to wait for chance happenings.

The German vinegar industry differs in many respects from the French because in Germany it is primarily beer and not wine that is used as the material for the manufacture of vinegar. A tall, hollow, wooden, well-ventilated pyramid is filled with beech shavings and a liquid containing alcohol is poured by drops into the upper opening. The vinegar bacteria live on all the surfaces of the shavings and the alcohol is all oxidized to acetic acid as the solution drips down through.

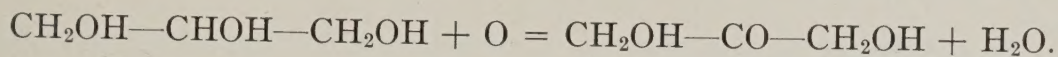
The Orleans method is most often used in Russia.

An interesting oxidation of polyvalent alcohols is carried out by some vinegar bacteria. *Bact. aceti* oxidizes mannite to fructose which is not oxidized further until all the mannite is consumed.¹ The oxidation effects of *Bact. xylinum* are still more remarkable.² These bacteria are able to change to a ketone group only the secondary alcohol group of each alcohol molecule. Thus sorbose is formed from sorbite, fructose from mannite and dioxycetone from glycerine.

¹ Brown, A. Jour. Chem. Soc. (London) 51: 638. 1887; Seifert, W. Centralbl. f. Bakt. (II) 3: 337. 1897.

² Bertrand, G. Compt. rend. 122: 900. 1896; 126: 653, 762, 842 and 984. 1898; 127: 124 and 728. 1898; 130: 1330. 1900; Idem. Bull. Soc. Chim. (3) 19: 302 and 347. 1898; Idem. Ann. Inst. Pasteur. 12: 385. 1898; Idem. Ann. de chim. et de physique (8) 3: 181. 1904; Bertrand named the bacteria which he studied "sorbose bacteria."

The latter reaction is important for the making of preparations of chemicals because it offers the only possibility of preparing this triose in a pure form:



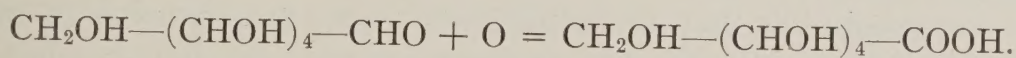
Purely chemical reactions always give a mixture of dioxyacetone $\text{CH}_2\text{OH}-\text{CO}-\text{CH}_2\text{OH}$ and glyceric aldehyde $\text{CH}_2\text{OH}-\text{CHOH}-\text{CHO}$, but in the case of biological oxidation by vinegar bacteria the primary hydroxyl group of the polyvalent alcohol is not attacked.

In the case of oxidation of the hexavalent alcohols, the vinegar bacteria always attack only the secondary α -hydroxyl group in the molecule, and then only if the secondary β -hydroxyl group has the same spatial arrangement of atoms as the α -hydroxyl group.¹ According to Bertrand, this is why these bacteria are unable to oxidize dulcitol although they readily convert sorbitol to sorbose.

All vinegar bacteria proceed to burn the acetic acid to CO_2 and H_2O if the alcohol is entirely consumed. This combustion of the acid does not occur in the presence of alcohol.

Under natural conditions, of course, the acetic acid fermentation is connected with the alcoholic fermentation. Here again we are dealing with the partnership of microorganisms already mentioned several times in this book. The fermentation bacteria have specialized so far that they join with one another and under certain conditions they may be mutually necessary. So it is in the case before us. By the presence of the vinegar bacteria the yeast is protected from an excess accumulation of alcohol which would retard the alcoholic fermentation. On their part, the vinegar bacteria require the alcohol formed by yeast to obtain their working energy in the process of acetic acid fermentation. It is therefore evident that acetic acid fermentation could not start on the earth earlier than alcoholic fermentation.

Bacteria have been described which effect a peculiar fermentation of sugar to gluconic acid:²



In this case the oxidation is limited to the union of one atom of oxygen with each molecule of sugar. According to the latest information,³ even the ordinary mold *Aspergillus niger* is able to effect this oxidation.

A specific microorganism has been discovered which splits ethyl alcohol into methane and CO_2 .⁴ The equation for this fermentation is not yet established but it is undoubtedly an oxidative fermentation.

In conclusion, it is to be emphasized that the oxidation of alcohol in an enzymatic process. Cell-free acetic acid fermentation proceeds only very

¹ Bertrand, G. *loc. cit.*

² Bouteux. *Compt. rend.* 91: 236. 1880.

³ Molliard, M. *Compt. rend.* 174: 881. 1922; 178: 41. 1924; Butkewitsch, W. *Biochem. Zeitschr.* 154: 177. 1924; Müller, D. *Ibid.* 199: 136. 1928; 205: 111. 1929; 213: 211. 1929.

⁴ Omeliansky, W. *Ann. Inst. Pasteur.* 30: 56. 1916.

weakly.¹ As a rule, the activity of the oxidizing enzymes is more effected on the average by killing the protoplasm than is the activity of the hydrolyzing enzymes.

20. THE CONNECTION BETWEEN THE CHEMICAL PROCESSES OF FERMENTATION AND OXYGEN RESPIRATION

All the above-described energizing processes of plants are very different at first glance. The material transformations in the different fermentations seem to have no connection with each other. Moreover, true fermentations cannot be carried out by purely chemical means, *i.e.*, without the participation of microorganisms. Up until recently the fermentations were also mysterious reactions in chemical respects. The process of oxygen respiration was likewise inconceivable because a total combustion of sugar to CO_2 and H_2O at the temperature of the surrounding air seemed paradoxical at first glance.

Recent biochemical investigations of the material transformations in fermentations and in oxygen respiration, however, have brought to light a remarkable relationship between all the energizing processes which take place at the expense of sugar. It will be clear from the following explanation that at present only a small part of the reactions concerned are understood, yet the results obtained are enough to give the outlines of the whole picture; we are therefore justified in speaking of a *single* fermentation in place of fermentations. On the other hand, a relationship has been established between fermentation and respiration; fermentation forms the first phase of ordinary oxygen respiration.

It was entirely natural to make a chemical study of alcoholic fermentation first because so many things favored such an investigation. The commercial importance of fermentation, the high rate of this process, the ease of preparation of considerable masses of yeast and of isolation of the active enzymes of yeast—all these conditions directed the attention of the biochemists to alcoholic fermentation, yet the achievements were less up until recently than in some other fields which were opened later to biochemical investigation. The reason for the slow progress is that alcoholic fermentation, as mentioned above, is a surprisingly well coördinated and regulated process. In fermentation there are only minute quantities of the secondary products which might indicate the nature of the cleavage of sugar. Sugar is so rapidly transformed to alcohol and CO_2 by fermentation that a quantitative method of determining sugar is based on the amount of carbon dioxide formed from it by yeast. The chemical investigation of fermentation accordingly has made progress only in recent times after new methods of artificial interference for the purpose of isolating the intermediate products of fermentation (p. 62) became available.

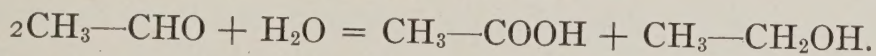
Although the genetic connection between respiration and fermentation was emphasized before the chemistry of fermentation was investigated, for the sake

¹ Buchner, E. und J. Meisenheimer. Ber. chem. Ges. 36: 624. 1903; Buchner, E. und K. Gaunt. Liebigs Ann. d. Chem. 349: 140. 1906; Rothenbach, F. und L. Eberlein. Deutschr. Essigind. 9: 233. 1905; Rothenbach und Hoffmann. Ibid. 11: 41 and 422. 1907; Idem. Zeitschr. f. Spiritusind. 30: 368. 1907.

of distinctness we shall next consider the chemical side of fermentation and later explain more fully the processes which take place during oxygen respiration.

21. THE INTERMEDIATE PRODUCTS OF ALCOHOLIC FERMENTATION

The first experiments to make clear the initial changes of the sugar molecule during alcoholic fermentation were unsuccessful because grape sugar can dissociate in different ways,¹ in connection with which the spatial arrangement of the atoms in the sugar molecule probably plays an important rôle. This presents great difficulties because the internal nature of spatial isomerism is still entirely unknown. New results were obtained after the foundation was laid for the experimental investigation of the following considerations: 1. Attention must first be given to the intermediate products which are related not to the material of fermentation (sugar) but to the end-products, and 2. An artificial interference in the process of fermentation is essential because this is the only way to obtain an appreciable amount of the intermediate products of fermentation.² The writer of this book was the first to move in this direction in that he published experiments,³ the keynote of which was that ethyl alcohol in fermentation is formed by reduction from acetaldehyde. The latter is therefore to be designated as the next-to-the-last product of alcoholic fermentation. It was observed that zinc and cadmium salts even in low concentrations strongly retarded the action of the reducing enzymes of yeast. On the basis of this finding, a fermentation was started with permanent yeast in the presence of these salts and it was actually found that the output of acetaldehyde became as much as a third of that of alcohol. This behavior of the zinc and cadmium salts is undoubtedly due to ionic reactions.⁴ Acetaldehyde is rapidly reduced to ethyl alcohol by yeast.⁵ This reduction takes place either by the direct addition of hydrogen⁶ or by transmutation into ethyl alcohol and acetic acid according to the Cannizzaro reaction:⁷



The Cannizzaro reaction, which was demonstrated in this connection for the first time in the plant kingdom, makes it easy to understand the frequent occurrence of acetic acid as a by-product. It was then proved that yeast is able to reduce all the aldehydes to the corresponding alcohols.⁸ Therefore this represents one of general reactions of the aldehydes.

¹ Nef, J. U. *Liebigs Ann. d. Chem.* 403: 204. 1914.

² Kostytschew, S. *Ber. chem. Ges.* 45: 1289. 1912; *Idem.* *Zeitschr. f. physiol. Chem.* 79: 130. 1912; 83: 93. 1913.

³ Kostytschew, S. und A. Scheloumow. *Ibid.* 85: 493. 1913; Kostytschew, S. und W. Brilliant. *Ibid.* 85: 493. 1913; Kostytschew, S. und L. Frey. *Ibid.* 111: 126. 1920; Kostytschew, S. und S. Zubkova. *Ibid.* 111: 132. 1920; *Idem.* *Jour. russ. bot. Ges.* 1: 47. 1916; 3: 40. 1918 (Russian); Kostytschew, S. *Biochem. Zeitschr.* 64: 237. 1914.

⁴ Kostytschew, S. und Zublowa. *loc. cit.*

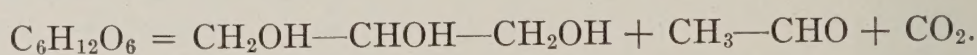
⁵ Kostytschew und Hübner. *Zeitschr. f. physiol. Chem.* 79: 359. 1912; Kostytschew, S. *Ibid.* 85: 408. 1913.

⁶ Kostytschew. *Ibid.* 92: 402. 1914.

⁷ Kostytschew. *Ibid.* 89: 367. 1914.

⁸ Neuberg, C. und Steenbock. *Biochem. Zeitschr.* 52: 494. 1913; 59: 188. 1914; Neuberg, C. und F. Nord. *Ibid.* 67: 24. 1914; Neuberg, C. und Schwenk. *Ibid.* 71: 114. 1915; Mayer, P. und C. Neuberg. *Ibid.* 71: 174. 1915; and others.

Other striking proofs have been produced recently of the intermediate formation of acetaldehyde during alcoholic fermentation. As early as the second year of the World War, Connstein and Lüdecke¹ patented a method of preparing glycerine from sugar by the action of yeast. This method is based on the idea that the active hydrogen necessary to reduce the aldehyde under normal conditions, can reduce to glycerine one of the "three-carbon substances" (see below) formed at an earlier phase of the fermentation, providing the acetaldehyde is removed. In order to do this, sodium sulphite is added to the fermenting liquid. The aldehyde is thereby fixed in the form of bisulphite compounds and an equi-molecular amount of glycerine is formed. The nature of the new method of producing glycerine was published after the World War and the process of "sulphite fermentation" was studied more closely. According to recent quantitative determinations,² the following equation is correct for sulphite fermentation:



The effect of sulphites and bisulphites on the accumulation of acetaldehyde in the process of fermentation was also independently discovered and described in the laboratories of the writer of this book³ and of a few other investigators.⁴ The principle of the sulphite method is different from that of the effect of zinc and cadmium salts, since the effect of the salts is directed to the reducing enzymes while the action of sulphite is on the aldehyde itself. In this case therefore, the reducing power of the yeast is not diminished but only directed to substances in another way.⁵ The increased formation of aldehyde in the presence of sulphites takes place not only during the fermentation of glucose or cane sugar but also during that of galactose.⁶

The third method of accumulating aldehyde is to modify the process of fermentation by adding methylene blue,⁷ which fixes the active hydrogen. Consequently part of the aldehyde is not reduced and there is a formation of both free acetaldehyde and acetic acid or ester (probably by transmutation).

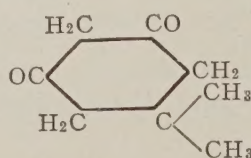
¹ Connstein, W. und K. Lüdecke. Ber. chem. Ges. 52: 1385. 1919; Zerner, E. Ibid. 53: 325 1920.

² Neuberg, C. und J. Hirsch. Biochem. Zeitschr. 100: 304. 1919; Neuberg, Hirsch und Reinfurth. Ibid. 105: 307. 1920.

³ Eliasberg, P. Jour. landwirt. Inst. in Petersburg, p. 74. 1921 (Russian).

⁴ Müller-Thurgau, H. und A. Osterwalder. Schweiz. landwirt. Jahrb., p. 408. 1915; Schweizer, K. Helv. chim. acta. 2: 167. 1919.

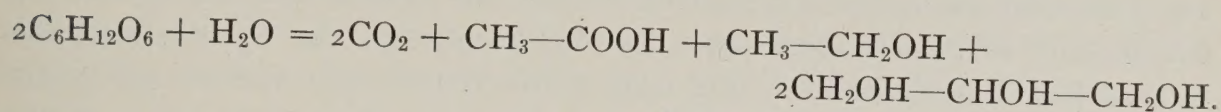
⁵ Since the action of zinc and cadmium salts touches only the dead yeast but that of sulphite the living yeast as well, the output of aldehyde is considerably greater in the latter case. The somewhat toxic sulphites could be replaced by dimethyl-cyclo-hexandion. Cf. Neuberg, C. und Reinfurth. Biochem. Zeitschr. 106: 281. 1920.



⁶ Tomita, M. Biochem. Zeitschr. 121: 164. 1921.

⁷ Kostytschew, S. Zeitschr. f. physiol. Chem. 83: 93. 1913.

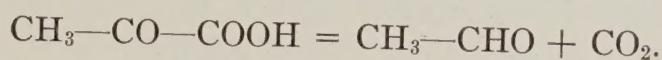
The fourth method of accumulating aldehyde is based on the effect of an alkaline reaction on yeast fermentation.¹ A considerable part of the resultant aldehyde is thereby changed to acetic acid and alcohol by the Cannizzaro reaction. The equation for the alkaline fermentation is described thus:²



Glycerine is therefore also formed.

A fifth method is very interesting because it gives the same general result as the sulphite method but it makes possible an intensive fermentation. There is an accumulation of acetaldehyde and glycerine in the presence of considerable quantities of animal charcoal.³ It is a remarkable condition that other adsorbents, such as diatomaceous earth, talc, and kaolin, are inactive. The mechanism of the effect of animal charcoal is still unexplained.

It is therefore clear that various kinds of information leave no doubt that acetaldehyde is an intermediate product of fermentation. This result is important inasmuch as it is the first case of the isolation of an intermediate product of such a biochemical process which cannot be carried out by purely chemical means. However, it is evident that this result does not make the nature of alcoholic fermentation entirely clear. The question arises, how can acetaldehyde be formed in the course of alcoholic fermentation? The writer of this book has assumed that acetaldehyde results from pyroracemic acid;⁴ the participation of this acid in the process of fermentation was previously explained as probable⁵ although at that time no experimental evidence could be produced in favor of this hypothesis. These experimental proofs have now been obtained because Neuberg and his co-workers have shown that pyroracemic acid is split into acetaldehyde and CO_2 by yeast:⁶



It is evident from this equation that the cleavage of pyroracemic acid is not a hydrolysis.

Experiments to capture the pyroracemic acid in the fermenting solution by means of CaCO_3 and similar substances⁷ proved unsuccessful.⁸ This is quite conceivable because both the salts and the bisulphite compound of

¹ Wilenko, G. G. *Ibid.* 98: 255. 1917; 100: 225. 1917; Oelsner, A. und A. Koch. *Ibid.* 104: 175. 1919.

² Neuberg, C. und J. Hirsch. *Biochem. Zeitschr.* 100: 304. 1919; Neuberg, Hirsch und Reinfurth. *loc. cit.*

³ Abderhalden, E. *Fermentforschung.* 5: 89, 110 and 225. 1921–1922; 6: 162. 1922; Abderhalden und Fodor. *Ibid.* 5: 138. 1919; Abderhalden und Glaubach. *Ibid.* 6: 143. 1922.

⁴ Kostytschew, S. *loc. cit.*

⁵ Neubauer und Fromherz. *Zeitschr. f. physiol. Chem.* 70: 350. 1910.

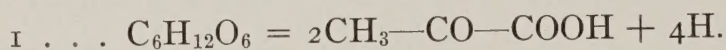
⁶ Neuberg, C. und Karczag. *Biochem. Zeitschr.* 36: 68. 1911; Neuberg, C. und Rosenthal. *Ibid.* 51: 128. 1912.

⁷ Fernbach et Schoen. *Compt. rend.* 157: 1478. 1913; 170: 764. 1920.

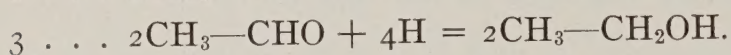
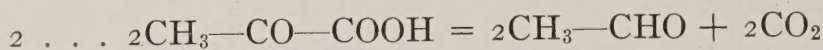
⁸ Kerb, J. *Ber. chem. Ges.* 52: 1795. 1919; *Idem.* *Biochem. Zeitschr.* 100: 3. 1919; Grab, M. v. *Ibid.* 123: 69. 1921; Kerb, J. und Zeckendorf. *Ibid.* 122: 307. 1921.

pyroracemic acid are decomposed by yeast in the same manner as the free acid, whereat not CO_2 and aldehyde but a carbonate or the bisulphite compound of acetaldehyde is formed. Only recently has the formation of pyroracemic acid during fermentation been demonstrated as follows.¹ To the fermenting sugar solution was added a semicarbazide salt, the solution was concentrated by evaporation after the sugar was entirely fermented, the pyroracemic-acid-semicarbazone precipitated in the form of its Ca or Zn salt and changed to the pyroracemic-acid-para-nitro-phenylhydrazone.

The last two coupled reactions of the process of fermentation are therefore now clear and the whole process can be represented schematically by the following steps:



Of course, this is a summarized equation of the first and so far uninvestigated part of alcoholic fermentation, and it very likely consists of several steps in itself. However, the others are simple chemical reactions:



It is to be emphasized expressly that a formation of intermediate products of fermentation with saturated carbon valences, such as acetaldehyde and pyroracemic acid, is unlikely for thermodynamic reasons. At the most, these substances only represent the stable forms into which the active intermediate products pass *under the influence of the trapping agencies*. We may regard as active intermediate products only unsaturated radicals, such as $\text{CH}_3\text{—CO—}$ and $\text{CH}_3\text{—CO—CO—}$ or $\text{CH}_3\text{—CO—CO—O—}$.

This is also supported by various facts. Thus it can scarcely be doubted that one of the very first stages in fermentation is the breaking of the hexose chain into two three-carbon chains. Still it is certain that the two trioses, which are said to be formed as saturated compounds, are not fermentable.² We must assume, therefore, that the molecule of sugar breaks up into unsaturated three-carbon complexes and the next transformation proceeds with a high velocity.

It is therefore clear that by the capture of intermediate products or rather (after the foregoing statement) secondary products, the chemical nature of fermentation can be recognized only in a rough way. For example, methyl glyoxal has been caught in some experiments in place of pyroracemic acid, which seems to be quite conceivable. If there is the unsaturated complex $\text{CH}_3\text{—CO—CO—}$, it depends entirely on the reduction potential at hand whether this complex takes up H or OH; methyl glyoxal is formed in the first case and pyroracemic acid in the second. A discussion of the transformation of methyl glyoxal into pyroracemic acid or of the reverse process would indicate

¹ Kostytschew, S. und Soldatenkow. Zeitschr. f. physiol. Chem. 176: 287. 1928.

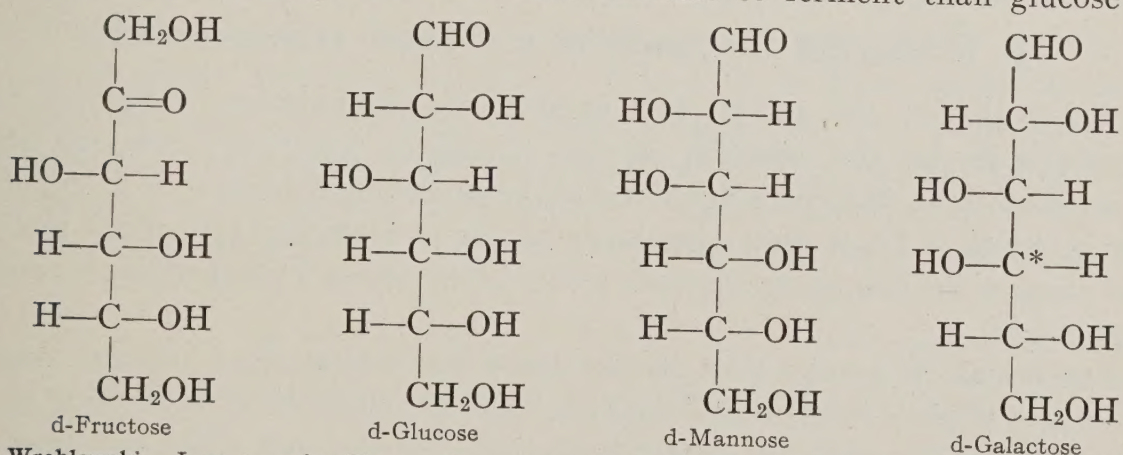
² Wohl. Ber. chem. Ges. 31: 1800. 1898; Neuberg, Färber, Levite und Schwenk. Biochem. Zeitschr. 83: 254. 1917; Haehn und Glaubitz. Ber. chem. Ges. 60: 490. 1927; Iwasaki. Biochem. Zeitschr. 203: 237. 1928; Kostytschew und Jegorowa. Zeitschr. f. physiol. Chem. 181: 264. 1929.

that one does not know how to estimate correctly the limits of error of the method of capture.

It would be very important to make clear the formation of the methyl group present in both acetaldehyde and pyroracemic acid. It is not improbable that the solution of this question will furnish the key to the whole problem of the process of fermentation. So far it has been possible to explain only to a certain degree the reason for the fermentability or the non-fermentability of the various sugars. The fermentable sugars do form with phosphoric acid or phosphates ester-like compounds, the so-called hexose phosphates, which are said to be necessary for the fermentation of sugar and are formed under the influence of the enzyme phosphatase of yeast.

It was emphasized long ago that phosphate hastens the fermentation of sugar to an extraordinary degree.¹ Then Harden and Young² proved a direct proportion between the output of CO₂ and alcohol and the amount of phosphate added. The inorganic phosphate is thereby changed to organic compounds of phosphorus.³ Two esters—hexose monophosphate⁴ and hexose diphosphate⁵ have been isolated. According to the view of most investigators, the latter compound is a secondary product which plays no rôle in fermentation.⁶

Attention should be given to the fact that only d-fructose is obtained from the cleavage of the hexose phosphate formed from different sugars.⁷ For this reason it appears that the fermentability of each sugar depends on whether it is possible to convert this sugar into a fermentable form, *e.g.*, into the γ -modification (p. 233), by way of hexose phosphate. If we compare the configuration of the various aldo-hexoses with that of d-fructose it is easy to see that both d-glucose and d-mannose are converted directly into d-fructose by merely changing the carbonyl group to the next-to-the-last carbon atom. The analogous transformation of galactose requires in addition a modification of the spatial arrangement of H and OH at the carbon atom marked with *. In connection with this, galactose is more difficult to ferment than glucose or



¹ Wroblewski. Jour. f. prakt. Chem. 64: 1. 1901; Idem. "Zymasegärung." 1903; and others.

² Harden and Young. Proc. Roy. Soc. London. 77: 405. 1906; 78: 369. 1906; 80: 299. 1908.

³ Harden and Young. Proc. Chem. Soc. 21: 189. 1905; 22: 283. 1906; Iwanoff, L. Arb. d. Naturforscherges. in Petersburg. Vol. 34. 1905 (Russian); Idem. Zeitschr. f. physiol. Chem. 50: 281. 1907.

⁴ Robison, R. Biochem. Jour. 16: 809. 1923.

⁵ Harden and Young. Biochem. Zeitschr. 32: 173. 1911; Young. Ibid. 32: 177. 1911.

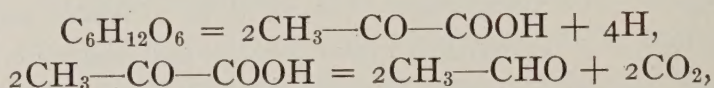
⁶ Kluyver and Struyk. Proc. Kon. Akad. Wet. Amsterdam. 29: 322. 1926.

⁷ Harden and Young. Proc. Roy. Soc. London. 77: 405. 1906; Neuberg, C. Biochem. Zeitschr. 88: 432. 1918.

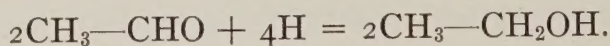
fructose. The other hexoses could be changed to d-fructose only by deep-rooted changes in configuration and are not fermentable. It is more difficult to explain the remarkable difference between α - and β -glucose; the former is more easily fermented by yeast.¹

The mechanism for all the above-described intramolecular rearrangements during the formation of the ester of phosphoric acid, has not been made clear. Hexose phosphate is also formed during the fermentation by living yeast² and it accumulates when the yeast cells are in a pathogenic (poisoned) condition. It must also be emphasized that the rôle of hexose phosphate cannot be defined more closely. The observation of Harden and Young may explain the significance of the spatial isomerism of the various hexoses but it does not explain the increase in the rate of fermentation by the addition of phosphates. Possibly the breaking of the hexose molecule is facilitated by the formation of the ester. Unfortunately the position of the phosphate in the hexose phosphate molecule has not yet been accurately established.

If we survey the experimental evidence in the field of the isolation of the intermediate products of fermentation, we come to the following conclusion. The formation, by certain influences, of pyruvic acid and of acetaldehyde in the process of fermentation has been established:



and in addition it has been proved that acetaldehyde is reduced to ethyl alcohol:



In the first stages of alcoholic fermentation is formed hexose monophosphate, the physiological rôle of which is still unknown. The other supposed products of fermentation and their probable transformations will be considered in the explanation of the theories of fermentation.

22. REDUCTION PROCESSES IN ALCOHOLIC FERMENTATION

Ethyl alcohol, the end-product of alcoholic fermentation, is formed from acetaldehyde by the addition of two atoms of hydrogen. Various other processes of reduction were described in the preceding chapter. It is therefore well to learn to know better the inner nature of biological reductions and for this purpose the process of alcoholic fermentation offers a particularly favorable opportunity.

Reductions in yeasts have been known for a long time. Yeast reduces molecular sulphur to hydrogen sulphide,³ also selenium salts,⁴ methylene blue,⁵ nitrobenzene (to anilin),⁶ all aldehydes to alcohols, etc. The reduction of

¹ Willstätter und Sobotka. *Zeitschr. f. physiol. Chem.* 123: 164. 1922.

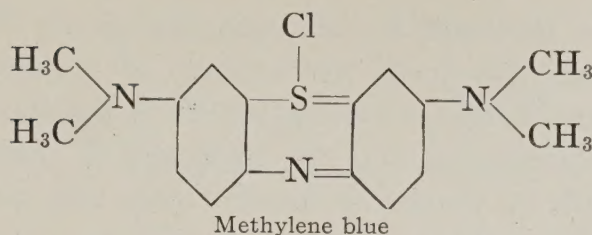
² Euler, H. v. and Johanssohn. *Zeitschr. f. physiol. Chem.* 80: 175. 1912.

³ Dumas. *Ann. de chim. et de physique.* 3: 57. 1874.

⁴ Palladin, W. *Zeitschr. f. physiol. Chem.* 56: 81. 1908.

⁵ "Zymasegärung," p. 343. 1903.

⁶ Pozzi-Escot. *Oxydases et reductases.* 1902; Neuberg, C. und E. Welde. *Biochem. Zeitschr.* 60: 472. 1914.



methylene blue, a compound with no oxygen, and of molecular sulphur shows that biological reduction is not based on the removal of oxygen but on the activation of hydrogen.

The rate of the addition of H can be quantitatively measured by determining the amount of reduced "hydrogen acceptors" (substances which readily take on hydrogen) of high molecular weight, such as methylene blue¹ or meta-dinitrobenzene.² (Methylene blue is easily titrated with titanium chloride.) In this way it is possible to demonstrate a relationship of reduction with the process of alcoholic fermentation by yeast. If the hydrogen of the fermenting solution is captured by means of methylene blue, the output of CO₂ and alcohol diminishes as follows: a deficit of 1 mol of hydrogen produces a shortage of 1 mol of CO₂ or alcohol.³ It is interesting that 1 mol of phosphoric acid causes the addition of 1 atom of hydrogen to methylene blue. From this it is evident that phosphates participate in the rearrangements of hydrogen during fermentation. It is remarkable that mannite and glycerine, of themselves non-fermentable, are fermented to CO₂ and alcohol by yeast in a rapid current of air.⁴ It might be assumed that 2 hydrogen atoms of mannite are removed, whereat the latter changes to a hexose.

It is therefore clear that there is a rearrangement of hydrogen in the reduction processes of yeast. It has long been thought⁵ that the reduction processes in yeast cells are produced and regulated by specific enzymes. At the present time Bach⁶ represents the mechanism of the action of reducing enzymes, or the so-called reductases, as follows. The substance which splits off hydrogen, Bach calls the reductor RH₂; the substance which takes on the hydrogen is given the name "hydrogen acceptor." According to Bach, aldehydes act as co-enzymes, since the reduction is often brought about by the hydrogen of water in that the oxygen of the water oxidizes the aldehyde and the hydrogen is activated by the enzyme perhydridase. Another theory, that of Wieland, will be considered later because it deals with the nature of oxygen respiration.

¹ Lwow, S. Mitt. russ. Akad. d. Wiss., p. 241. 1913; p. 1171. 1915 (Russian); *Idem.* Biochem. Zeitschr. 66: 440. 1914; *Idem.* Tageb. d. 1 Kongr. russ. Bot., p. 61 and 62. 1921 [Russian].

² Lipschitz, W. und A. Gottschalk. Pflügers Arch. f. ges. Physiol. 191: 1 and 51. 1921.

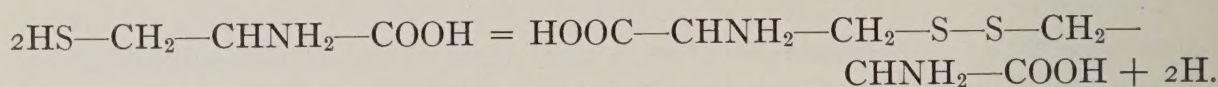
³ Lwow, S. *loc. cit.*

⁴ Kostytschew, S. und Faermann. Zeitschr. f. physiol. Chem. 173: 72. 1928.

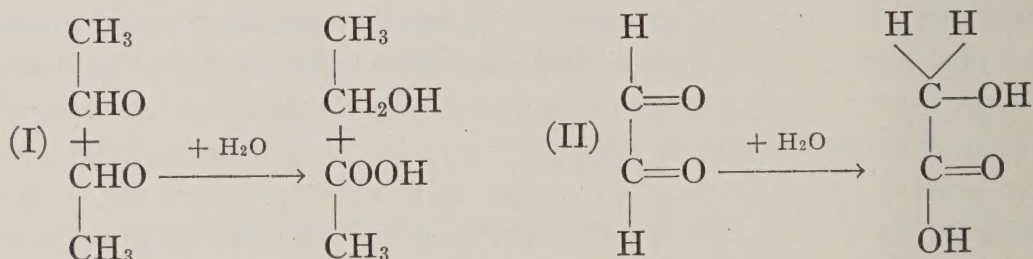
⁵ Rey-Pailhade. Compt. rend. 106: 1683. 1888; *Idem.* Bull. Soc. Chim. de France (3) 23: 666. 1900; Pozzi-Escot. *loc. cit.*; Schardinger. Zeitschr. f. Untersuch. Nahrungs- und Genussmittel. 5: 22. 1902.

⁶ Bach, A. Arch. sci. phys. et nat. Genève (4) 32: 27. 1911; Biochem. Zeitschr. 31: 433. 1911; 33: 282. 1911; 38: 154. 1911; 52: 412. 1913; 58: 205. 1913; Arch. sci. phys. et nat. Genève (4) 43: 307. 1917; Compt. rend. 164: 248. 1917; see also Euler, H. v. und R. Nilsson. Zeitschr. f. physiol. Chem. 149: 44. 1925; 151: 155. 1926; 152: 264. 1926; Haehn und Pülz. Chem. d. Zelle und Gewebe. 12: 65. 1924.

A supposed source of hydrogen is the sulphydryl group SH ,¹ in that all compounds which contain this group are capable of polymerization: the latter process consists in the linkage of two molecules of the substance concerned by means of their sulphur atoms and the hydrogen is thereby split off.^u For example, one molecule of cystine is formed from two molecules of cysteine.



The Cannizzaro reaction is also by nature a transfer of atomic hydrogen. According to the view of the author of this book, there is no sharp distinction between aldehyde reduction *sensu stricto* and the transmutation according to Cannizzaro. On the other hand, the Cannizzaro reaction (I) is entirely analogous to both the rearrangement of glyoxal to glycolic acid (II) and the formation of benzylic acid from benzyl (III). All three types of reaction involve a "displacement" of hydrogen or of a radical to the adjacent carbon atom and therefore might be effected by a single enzyme.

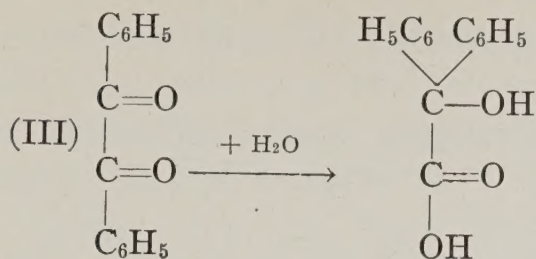


¹ **Heffter.** Arch. f. exp. Path., Festschr. f. Schmiedeberg, p. 253. 1908; **Strassner.** Biochem. Zeitschr. 29: 295. 1910; **Meyerhof.** Pfügers Arch. f. ges. Physiol. Vol. 169. 1917; 170: 367 and 428. 1918; Vol. 175. 1919; Zeitschr. f. physiol. Chem. 101: 165. 1918; 102: 1. 1918.

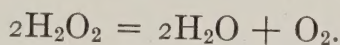
^u This point and all related questions has been studied most thoroughly in connection with glutathione, the substance which has been found in both plant and animal tissues and which acts as either an acceptor or a donator of hydrogen according as it is present in the oxidized or reduced form. It is especially abundant in yeast and it was from this plant that **Hopkins** (Biochem. Jour. 15: 286-305. 1921) first isolated and described it. The name glutathione was given it because analysis indicated that it was a union of α -amino glutaric acid and α -amino, β -thiopropionic acid and for some time it was generally thought that the synthesis of this dipeptide by **Stewart** and **Tunncliffe** (Biochem. Jour. 19: 207. 1925) and the close resemblance between the synthetic and natural products had established the point beyond all doubt. Other workers proceeded to find glutathione in various plant and animal tissues. **Hopkins** (Biochem. Jour. 19: 787-819. 1925) found a close relationship between it and the oxidation of fats and proteins while **Harrison** (Biochem. Jour. 18: 1009. 1924; 21: 335 and 1404. 1927) showed that its action depended on the catalytic action of traces of iron.

Typical examples of the demonstration of glutathione in plant tissues are those by **Kozlowski** (Biochem. Jour. 20: 1346. 1926), **Camp.** (Science. 69: 458. 1929) and **White, V. B.** (*Ibid.* 71: 74-76. 1930). Both Camp and Miss White used **Fink's** micro-method (Science. 65: 143. 1927) which depends on a flash reaction of red or pink with a nitro-prusside reagent and these workers agree that the presence of glutathione can best be demonstrated in meristematic tissues.

Because of adverse criticism by a few investigators (*cf.* **Hunter** and **Eagles.** Jour. Biol. Chem. 72: 147. 1927) concerning the exact composition of preparations of glutathione from yeast and animal tissues, **Hopkins** (Jour. Biol. Chem. 84: 269-320. 1929) made a reinvestigation of glutathione from yeast and found that it is a tripeptide of glycine, glutamic acid and cysteine, rather than a dipeptide. An almost simultaneous confirmation of this new analysis was reported by **Kendall, McKenzie** and **Mason** (*Ibid.* 84: 657-674. 1929) who have suggested a formula for the new compound which has been obtained in pure crystalline form. It will now be necessary to confirm the presence of "glutathione" in other tissues from which it has been reported but it is very probable that the revision of the constitution of this peculiar substance will have little effect upon such reports except that a dependence on iron or other metallic catalysts may be brought out.—*Ed.*



On the basis of the foregoing explanation, it is clear that the reduction processes of yeast are related to the alcoholic fermentation and are due to a transfer of atomic hydrogen. In addition, yeast contains a large amount of catalase, an enzyme which decomposes hydrogen peroxide into water and oxygen:¹



The significance of catalase is not understood; it has not even been decided that it is an independent enzyme. Indeed the assumption is not precluded that the decomposition of hydrogen peroxide is only a secondary reaction in the action of other enzymes. It is therefore hardly advisable to set up hypotheses concerning the rôle of catalase in the process of fermentation.

23. THEORIES OF ALCOHOLIC FERMENTATION

The oldest chemical theory of fermentation is that of A. v. Baeyer.² It states that there are repeated additions and removals of water in the glucose molecule whereby there is finally formed a six-membered chain with oxidized carbon atoms in its middle. This is not resistant and is broken either at one point or at three different places; in the first case, two molecules of lactic acid are formed but otherwise alcohol and carbon dioxide. The following equations represent the gradual alteration of the sugar molecule according to Baeyer:

- I. $\text{CH}_2\text{OH}-\text{CHOH}-\text{CHOH}-\text{CHOH}-\text{CHOH}-\text{CH}_2(\text{OH})_2$ (glucose hydrate),
- II. $\text{CH}_3-\text{CHOH}-\text{C}(\text{OH})_2-\text{C}(\text{OH})_2-\text{C}(\text{OH})_2-\text{CH}_3$,
- III. $\text{CH}_3-\text{CHOH}-\text{CO}-\text{O}-\text{CO}-\text{CHOH}-\text{CH}_3$,
- IV. $\text{CH}_3-\text{CH}-\text{O}-\text{CO}-\text{O}-\text{CO}-\text{O}-\text{CH}_2-\text{CH}_3$.

This theory is not supported by experimental investigations but it contains the correct idea that alcoholic fermentation and lactic acid fermentation are identical in their first stages and that an intramolecular shifting of hydrogen takes place during fermentation.

The theory of Buchner and Meisenheimer³ assumes that alcohol is formed by way of the intermediate stage of lactic acid. These investigators therefore believe that they can show that lactic acid fermentation precedes alcoholic fermentation. According to them, lactic acid is formed from sugars by way of triose and methyl glyoxal $\text{CH}_3-\text{CO}-\text{CHO}$ in the following manner:⁴

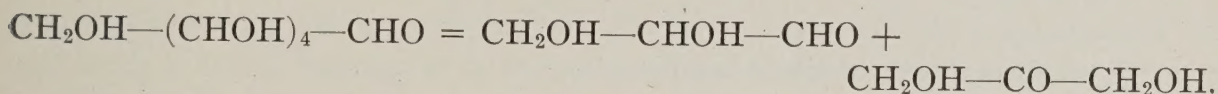
¹ Loew, O. Report of U. S. Dept. Agric. No. 68: 1901; Issajew. Zeitschr. f. physiol. Chem. 42: 102. 1904; 44: 546. 1905.

² Baeyer, A. v. Ber. chem. Ges. 3: 63. 1870.

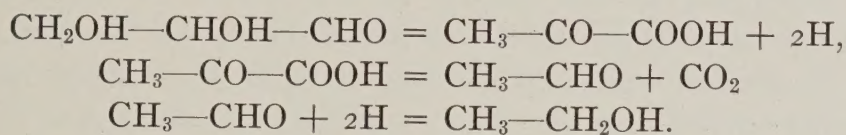
³ Buchner, E. und J. Meisenheimer. Ber. chem. Ges. 37: 417. 1904; 38: 620. 1905.

⁴ Wohl. Biochem. Zeitschr. 5: 45. 1907; see also Nef. Liebigs Ann. d. Chem. 335: 247. 1904; Buchner, E. und Meisenheimer. Ber. chem. Ges. 43: 1773. 1910.

alcohol by atomic hydrogen. This is the first time that the reducing properties of yeast have been taken into account in a theory of fermentation. However, in this theory there are no hypotheses concerning the process of forming pyroracemic acid from the sugar. This is explained in the following theories. A. v. Lebedew¹ first made the assumption that the hexose molecule breaks up into one molecule of glyceric aldehyde and one of dioxyacetone in the first phase of fermentation:

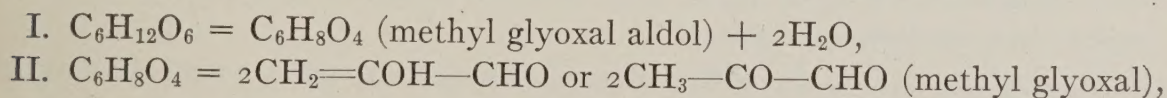


Later glyceric aldehyde is said to yield alcohol and CO_2 according to the foregoing theory of Kostychev:



Dioxyacetone unites with phosphates, according to Lebedew, and forms triose monophosphate, which then condenses to hexose diphosphate. Sugar is said to be regenerated from the latter and the whole process is repeated until all the sugar is broken down into CO_2 and alcohol by way of glyceric aldehyde. However, the experimental basis of this theory is erroneous because it has been found that no triose monophosphate is formed during the fermentation of dioxyacetone.² The author himself therefore abandoned his first theory and proposed another one.³ The new theory was based on the fact that both glyceric aldehyde and glyceric acid are fermentable, according to the author's experiments. Lebedew assumes that the fermentation of sugar takes place by way of the intermediate stages of glyceric aldehyde, glyceric acid and pyroracemic acid. This theory is likewise erroneous because neither glyceric aldehyde nor glyceric acid is fermentable.⁴

Neuberg⁵ proposed a theory in which the Cannizzaro reaction, first noticed in yeast by Kostychev, plays a prominent rôle. According to this theory, there are no trioses formed during fermentation and the molecule of sugar breaks up into two molecules of methyl glyoxal. Pyroracemic acid is then formed by the transmutation of methyl glyoxal according to Cannizzaro. Later there is a cleavage of the pyroracemic acid into acetaldehyde and CO_2 , but finally there is a "mixed" Cannizzaro reaction between methyl glyoxal and acetaldehyde whereby ethyl alcohol is formed and pyroracemic acid is regenerated. These involved processes can be elucidated by the following equations:



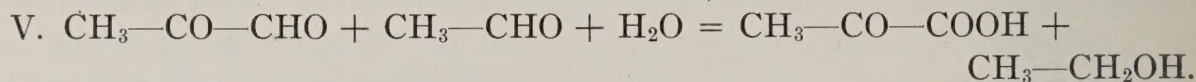
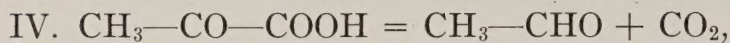
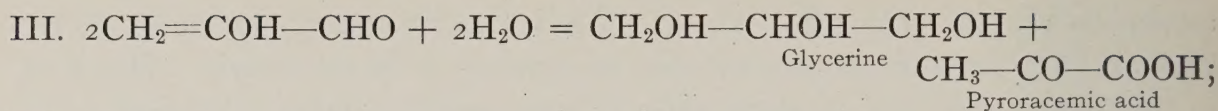
¹ Lebedew, A. v. *Compt. rend.* 153: 136. 1911; *Bull. Soc. Chim. de France* (4) 9: 678. 1911; *Ber. chem. Ges.* 45: 3240. 1912; *Investigations in cell-free alcoholic fermentation.* 1913 (Russian).

² Neuberg, C., Färber, Levite und Schwenk. *loc. cit.*

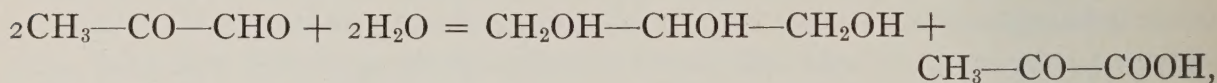
³ Lebedew, A. v. *Zeitschr. f. physiol. Chem.* 132: 275. 1924; Lebedew and Polonsky. *Jour. russ. chem. Ges.* 49: 344. 1917.

⁴ Cf. citations in note 1 on the preceding page.

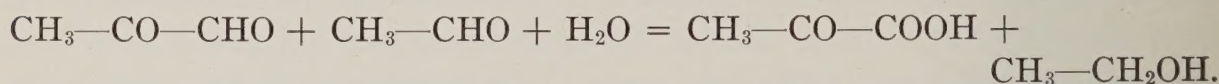
⁵ Neuberg, C. und Kerb. *Biochem. Zeitschr.* 53: 406. 1913; 58: 158. 1913.



This theory tries primarily to make clear the formation of the methyl groups in the process of fermentation. In the molecule of methyl glyoxal there are various possible intramolecular rearrangements of atoms and the formula of this compound may be written in various ways, *e.g.*, $\text{CH}_3-\text{CO}-\text{CHO}$, $\text{CH}_2=\text{COH}-\text{CHO}$, $\text{CH}_2=\text{COH}-\text{CH}(\text{OH})_2$, etc. Still Neuberg's theory is likewise untenable. According to this theory, all reductions are entirely the result of transmutation of prepared aldehydes according to the Cannizzaro reaction. Hence, two Cannizzaro reactions are said to take place simultaneously; the one between two molecules of methyl glyoxal (with the addition of two molecules of water), by which glycerine and pyruracemic acid are produced



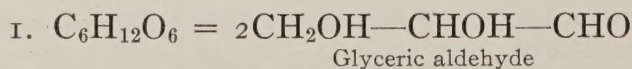
and the other between one molecule of methyl glyoxal and one of acetaldehyde, and only in the one direction:



Such a course of the process could be explained only by a regulatory principle which controls all the reactions, in addition to the purely enzymatic processes. Still we know that yeast changes acetaldehyde only to acetic acid and ethyl alcohol,¹ and that the Cannizzaro reaction between two aldehydes yields four products in place of two,² whereat the simple Cannizzaro reaction proceeds with greater rapidity than the mixed one. The even course of the mixed reaction is therefore scarcely conceivable.

In addition it must be borne in mind that methyl glyoxal is not fermentable; of course this experimental result cannot be put aside by theoretical explanations. As for the formation of glycerine, it could be formed by more simple processes. The sulphite fermentation does not support the Neuberg theory. This process can be explained by various theories because the hypothesis of the intermediate formation of a "three-carbon substance" is to be found in all modern theories of fermentation. Glycerine is probably a reduction product of these "three-carbon substances."

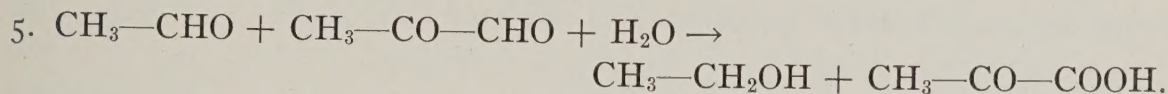
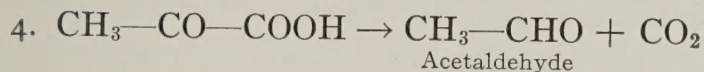
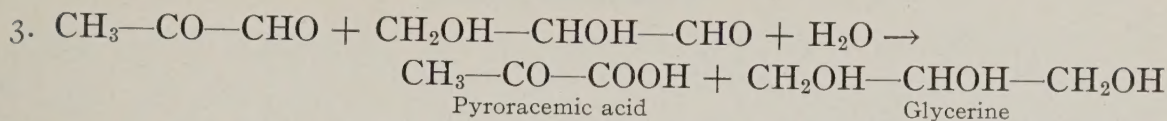
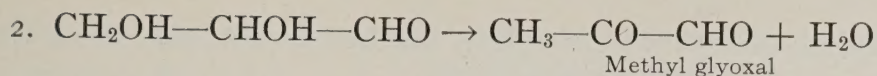
Faworsky³ represents the entire process of fermentation by means of successive additions of water and after that the following removals of water:



¹ Kostytschew, S. Zeitschr. f. physiol. Chem. 89: 367. 1914.

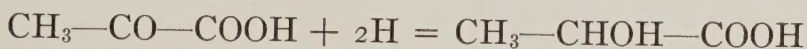
² Nord, F. F. Biochem. Zeitschr. 106: 275. 1920.

³ Faworsky, A. Bull. Soc. Chim. de France (4) 31: 216. 1926.

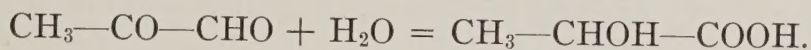


This theory also requires some regulation because a simultaneous addition and removal of water is impossible (*ausgeschlossen*) in a homogeneous medium.

On the basis of these statements, we can, however, understand all the secondary products of alcoholic fermentation. Glycerine is formed from a reducible "three-carbon substance." Lactic acid is either a reduction product of pyroracemic acid:



or a transformation product of methyl glyoxal:



Acetic acid is formed from acetaldehyde by transmutation according to the Cannizzaro reaction.

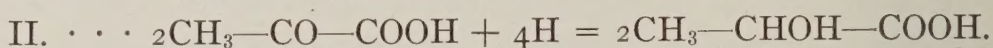
Not one of the present theories of fermentation is correct. They all contradict either the experimental results of various investigators or the current principles of chemistry. Above all, however, it must be emphasized again that a formation of intermediate products with saturated carbon valences is hardly conceivable. On the other hand it is scarcely possible to build up a scheme for fermentation without bearing in mind physico-chemical motives. The theories proposed by pure organic chemists are unlikely for the following reasons. In the first place, they contradict thermodynamic principles in so far as they presuppose only saturated intermediate products. Secondly, these theories involve assumptions of processes which are impossible in a structureless and even homogeneous medium. For example, they include several simultaneous Cannizzaro reactions or simultaneous additions and removals of water. They should at least provide for physico-chemical conditions under which the supposed reactions could proceed in the assumed direction.

In this connection, cell-free fermentation must be mentioned again. If it is assumed that each step in fermentation is effected by a particular enzyme, it must be supposed that there is the possibility of a regulation which is unrelated to structure. Such a vitalistic hypothesis is now unfounded. On the contrary, if we assume the existence of a single enzyme of fermentation, we must remember that a spontaneous splitting of sugar in the sense of alcoholic fermentation is unproved; but an enzyme is only a catalyst of a spontaneous reaction. In spite of this, the hypothesis of a single enzyme is always the more probable.

24. THE INTERMEDIATE PRODUCTS AND THE CHEMICAL NATURE OF OTHER FERMENTATIONS

On the basis of the foregoing statements, the relationship of the other fermentations is rather evident. It was pointed out on the preceding pages that Buchner and Meisenheimer¹ discovered a slight formation of lactic acid during alcoholic fermentation and therefore regarded lactic acid fermentation as the stage before alcoholic fermentation. Palladin² obtained a fermentation of small quantities of lactic acid to CO₂ and alcohol by yeast and accordingly accepted the theory of Buchner and Meisenheimer in general. Microörganisms have also been described which oxidize lactic acid to pyroracemic acid.³

However, the more probable hypothesis is the opposite one—that pyroracemic acid is the common intermediate product of alcoholic fermentation and lactic acid fermentation—but against this assumption is the fact that lactic acid is not affected by the enzymes of alcoholic fermentation. The same cleavage of sugar into pyroracemic acid and hydrogen might take place in both alcoholic and lactic acid fermentation. Later the pyroracemic acid would be broken up into CO₂ and acetaldehyde in the process of alcoholic fermentation, but in lactic acid fermentation would at once be reduced to lactic acid by the active hydrogen. The bacteria of pure lactic acid fermentation are unable to split pyroracemic acid.⁴ Therefore the hydrogen does not reduce the carbonyl group of acetaldehyde but that of pyroracemic acid:



Kostytchev and Soldatenkow⁵ have caught pyroracemic acid in pure lactic acid fermentation, by means of the semicarbazide method.^v

Still clearer is the connection between lactic acid fermentation and alcoholic fermentation if other products of fermentation are formed in addition to lactic acid. For example, *Bacterium lactis aerogenes* produces considerable quantities of acetic acid and ethyl alcohol. Both these products come from acetaldehyde and a considerable yield of aldehyde has actually been obtained by the sulphite fermentation of this organism.⁶ In the case of impure lactic acid fermentation, then, part of the pyroracemic acid that is formed is not reduced but split. In judging the relationship between alcoholic fermentation and lactic acid fermentation, it is also to be taken into consideration that, according to the latest informa-

¹ Buchner, E. und J. Meisenheimer. Ber. chem. Ges. 37: 417. 1904; 38: 620. 1905. See also Fernbach, A. et M. Schoen. Compt. rend. soc. de biol. 89: 475. 1923.

² Palladin, W., Sabinin and Lowtschinowsky. Mitt. russ. Akad. Wiss., p. 701. 1915 (Russian); Palladin and Sabinin. Ibid., p. 187. 1916 (Russian); Idem. Biochem. Jour. 10: 185. 1916.

³ Mazé, P. Bull. Soc. de Biol. 81: 1150. 1918.

⁴ Kostytshew, S. und M. Afanassjewa. Compt. rend. July, 1925.

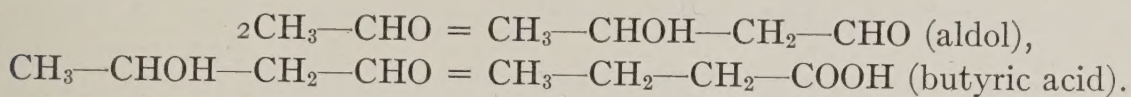
⁵ Kostytshew, S. und S. Soldatenkow. Zeitschr. f. physiol. Chem. 168: 124. 1927.

^v It must be noted in passing, however, that Neuberg, C. and Kobel (Biochem. Zeitschr. 207: 232–262. 1929) have reported definitely that they have isolated methyl glyoxal in lactic acid fermentation. For the steps in the conversion to lactic acid see p. 436.—Ed.

⁶ Neuberg, C., F. F. Nord und E. Wolff. Biochem. Zeitschr. 112: 144. 1920; Nagai, K. Ibid. 141: 261. 1923.

tion, the typical lactic acid bacteria, *Bact. lactis aceti* Leichm., like yeast, can form hexose phosphate.¹

Butyric acid fermentation gives us another picture. The assumption was made long ago that butyric acid is a secondary synthetic product and that it is formed from two molecules of acetaldehyde by way of aldol:²

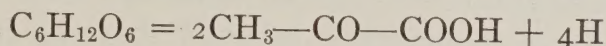


The conversion of aldol into butyric acid has been produced by purely chemical means and the use of silent electrical discharges.³

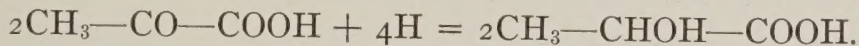
In the case of butyric acid fermentation, the principal by-products are lactic acid, acetic acid and ethyl alcohol, which brings to light the genetic relationship of butyric acid fermentation with alcoholic and lactic acid fermentation. Acetaldehyde has been captured during butyric acid fermentation by the use of the sulphite method.⁴ On the other hand, butyric acid is formed during the fermentation of pyroracemic acid aldol by means of the bacteria of butyric acid fermentation.⁵

On the basis of the evidence just described, it is possible to form a simple, connected picture of the chemical unity of all of the three true fermentations, if we adopt the hypothesis of the author of this book concerning the importance of active hydrogen in the process of fermentation. First there is always the same cleavage of sugar into pyroracemic acid and active hydrogen; the next step depends on whether the addition of hydrogen or other simultaneous processes go the faster. Either lactic acid or alcoholic or butyric acid fermentation is then started, depending on the ratio of the reaction velocities.

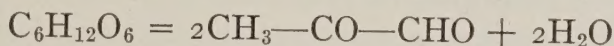
First Case. After the summarized reaction



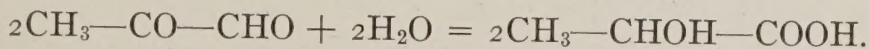
the rate of cleavage of pyroracemic acid is considerably less than that of the taking up of hydrogen. This results in the reduction of the pyroracemic acid or, in other words, there is lactic acid fermentation:



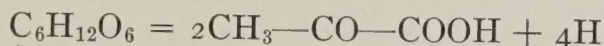
The lactic acid might also eventually be formed from methyl glyoxal



and later



Second Case. After the summarized reaction



¹ Virtanen, A. J. Zeitschr. f. physiol. Chem. 138: 136. 1924; 143: 71. 1925.

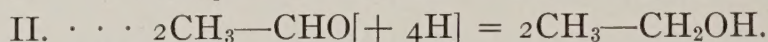
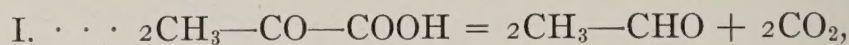
² Fitz, A. Ber. chem. Ges. 9: 1348. 1876; 11: 42. 1878; Buchner, E. und J. Meisenheimer. Ibid. 41: 1410. 1908.

³ Loeb, W. Biochem. Zeitschr. 20: 126. 1909.

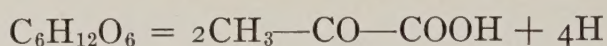
⁴ Neuberg, C. und F. F. Nord. Biochem. Zeitschr. 96: 143. 1919.

⁵ Neuberg, C. und B. Arinstein. Ibid. 117: 269. 1921.

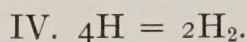
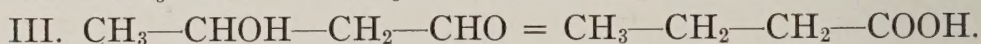
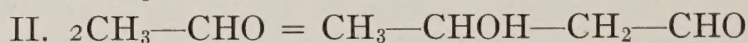
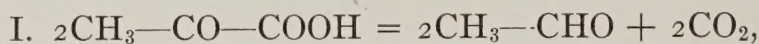
the rate of cleavage of pyroracemic acid is considerably greater than that of the taking up of hydrogen. Consequently not pyroracemic acid but acetaldehyde is reduced by the active hydrogen and the whole process is therefore nothing but alcoholic fermentation:



Third Case. After the summarized reaction

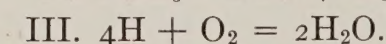
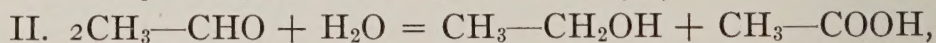
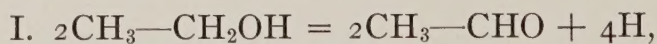


the processes of reduction proceed very slowly. Before the carbonyl groups present are reduced, there is a formation of butyric acid and the disappearance of the carbonyl groups. Once this done, it is no longer possible to add on the hydrogen since there are no free carbonyl groups available. Consequently there is a liberation of molecular hydrogen:



The so-called impure fermentations which were considered above, are various transitions between these three types of reactions. Such a simple relationship between the reactions which take place in all the fermentations is possible only by assuming a jumping (*Überspringen*) of the hydrogen in the process of fermentation (p. 434), in accord with the ideas of the author of this book. If it is assumed, as Neuberg does, that all the reductions of carbonyl groups are only Cannizzaro reactions, it is impossible to express the unity of the process of fermentation by the simple equations indicated in the foregoing scheme. Of course, it is to be borne in mind that the foregoing hypotheses only very approximately represent the true facts, because the saturated steps, such as pyroracemic acid and acetaldehyde, are actually not formed (*cf.* p. 430).

Acetaldehyde is also produced during acetic acid fermentation, as Pasteur has already emphasized. Considerable quantities of acetaldehyde have been captured during the fermentation of *B. orleanense*, *B. ascendens* and *B. Pasteurianum*,¹ by the use of the sulphite method. According to current ideas, the oxidation of alcohol to acetaldehyde is not the result of the activation of oxygen but of hydrogen. The entire process probably consists, then, of three stages:



The following circumstance shows that such an explanation is correct. It is possible to start acetic acid fermentation with oxygen excluded in the presence of a suitable hydrogen acceptor, such as methylene blue.² From this it is evident that the oxygen functions merely as a hydrogen acceptor.

¹ Neuberg, C. und F. F. Nord. *Biochem. Zeitschr.* 96: 158. 1919.

² Wieland. *Ber. chem. Ges.* 46: 3327. 1913.

25. ANAEROBIC RESPIRATION

The genetic relationship between all the kinds of fermentation, explained on the preceding pages, becomes still more significant when it is taken into consideration that the normal oxygen respiration of plants is likewise genetically connected with fermentation. In order to explain this matter thoroughly it is necessary to describe some facts that were discovered long ago but correctly interpreted only recently.

During his investigations of alcoholic fermentation, Pasteur¹ made an important discovery, the full significance of which was made clear only in the present century. It was found that various plants and even separate parts of plants regularly produce CO₂ and a certain amount of alcohol with oxygen excluded. Pasteur concluded from this that under certain conditions anaerobic processes also play an important rôle in the normally respiring forms of life. At that time it was impossible to obtain a deeper insight into the nature of the phenomena concerned.

The liberation of CO₂ by normally respiring plants with oxygen excluded, which later came to be known as anaerobic respiration,² was the object of numerous experimental investigations. Above all, it is to be noted that the rate of anaerobic respiration in most cases is considerably less than that of oxygen respiration. The output of energy by the fermentation of a certain amount of sugar is only a small fraction of the energy of respiration of the same amount of sugar (p. 396); consequently most aerobic plants rapidly become exhausted in the absence of oxygen, on account of lack of working energy.

The following table from Wilson³ illustrates the ratio of the rate of anaerobic respiration to that of oxygen respiration for various plants. This ratio is usually

expressed as $\frac{I}{N}$.

	$\frac{I}{N}$
Seedlings of <i>Triticum vulgare</i>	0.49
Seedlings of <i>Sinapis alba</i>	0.18
Seedlings of <i>Brassica napus</i>	0.25
Seedlings of <i>Helianthus annuus</i>	0.35
Stem of <i>Orobancha ramosa</i>	0.32
Leafy shoots of <i>Abies excelsa</i>	0.08
Fruiting bodies of <i>Lactarius piperatus</i>	0.32

It is evident that such a weak fermentation can furnish very little energy, as experiments actually show.⁴ On the other hand, it was found that anaerobic

¹ Pasteur, L. Compt. rend. 75: 784. 1872; Etudes sur la bière. 1876; cf. also Lechartier et Bellamy. Compt. rend. 69: 356 and 466. 1869; 75: 1203. 1872; 79: 1006. 1874.

² Kostytschew, S. Investigations of the anaerobic respiration of plants. 1907 (Russian).

³ Wilson. Flora. 65: 93. 1882; cf. also Möller. Ber. bot. Ges. 2: 306. 1884.

⁴ Eriksson. Untersuch. d. bot. Inst. Tübingen. 1: 636. 1881-1885.

respiration is generally common in the plant kingdom; it occurs in both seed-plants and aerobic molds and in other normally respiring lower organisms.¹

In estimating the physiological rôle of anaerobic respiration, some workers have made the assumption that this process is a biological adaptation. The instantaneous death of plants when oxygen is accidentally withheld from them is said to be prevented by anaerobic respiration. Even though anaerobic respiration is insufficient as a constant source of energy, it might, according to this hypothesis, maintain the life of the plant for a few hours or even a day, and under certain conditions this might be very important.

This conception must be rejected as insufficient and disproved. Not to mention that biological-teleological considerations are inadmissible as a rule in biochemical questions, the supposition is in itself hardly plausible that a physiological process would appear only incidentally as a precautionary measure. Indeed we know that anaerobic respiration is universally widespread. So far there has not been found a single plant which is unable to form CO_2 in the absence of oxygen.^w On the other hand, anaerobic respiration occurs very seldom under natural living conditions. In what manner could a rarely occurring process attain the standing of a widespread phenomenon of adaptation? Finally, it is to be taken into consideration that plants do not die instantly without forming CO_2 . The energy value of anaerobic respiration is, however, at best so insignificant that no biological usefulness can be granted to it. It is actually questionable whether the scanty gain of energy supplied by anaerobic respiration can delay the death of the plant even for a short time.

The influence of external factors on anaerobic respiration is the same as in the case of normal respiration.² Anaerobic respiration is also often identical with alcoholic fermentation; however, it was shown above that the external factors exert the same effect on both oxygen respiration and alcoholic fermentation. A quantitative determination of the balance of anaerobic respiration was first carried out with germinating seeds;³ it was found that the ratio of CO_2 to alcohol for pea seeds corresponded approximately with the theoretical equation for alcoholic fermentation. Acetaldehyde was captured by the sulphite method in the anaerobic respiration of pea seeds.⁴ The same result was obtained with molds which respire normally in the presence of oxygen, after confining them in a medium free from oxygen.⁵ After obtaining these quantitative determinations

¹ Muntz. Ann. de chim. et de physique (5) 8: 56. 1876; 13: 543. 1878; Brefeld, O. Landwirt. Jahrb. 5: 281. 1876; de Luca. Anr. sci. nat. (6) 6: 286. 1878.

^w An unconfirmed exception to this statement is found in *Elodea canadensis*, according to a few experiments by Lyon (J. Gen. Physiol. 6: 299-306. 1923-24).—Ed.

² Amm. Jahrb. wiss. Bot. 25: 1. 1893; Detmer. Ber. bot. Ges. 10: 201. 1892; Chudiakow. Landwirt. Jahrb. 23: 333. 1894; Palladin. Rev. gén. bot. 6: 201. 1894; Fernandes, D. S. Recueil des travaux bot. néerland. 20: 107. 1923; [Lyon. J. Gen. Physiol. loc. cit.; Karlsen, A. Amer. Jour. Bot. 12: 619-624. 1925.]

³ Godlewski, E. and Polzeniusz. Bull. de l'acad. des sci. de Cracovie, p. 267; 1897; p. 227. 1901.

⁴ Neuberger, C. und A. Gottschalk. Biochem. Zeitschr. 151: 167. 1924; [160: 256. 1925; Bodnar, Szepessy und Ferenczy. Ibid. 165: 16. 1925. For acetaldehyde in the anaerobic respiration of other higher plants see Thomas, M. Biochem. Jour. 19: 927. 1925; Klein und Pirschle. Biochem. Zeitschr. 168: 340. 1926; Harley and Fisher. Jour. Agr. Res. 35: 938-993. 1927.]

⁵ Kostytschew, S. Ber. bot. Ges. 25: 44. 1907; Kostytschew and Afanassjewa. Jour. russ. bot. Ges. 2: 77. 1917 (Russian); Idem. Jahrb. wiss. Bot. 60: 628. 1921.

we are justified in speaking of an identity of anaerobic respiration with alcoholic fermentation.

Later it was also shown¹ that the value of CO₂: alcohol during anaerobic respiration is not always in accord with the equation of alcoholic fermentation, and a considerable excess of CO₂ is often produced. In the case of a few plants there is no formation of alcohol when oxygen is excluded. These extreme cases can be explained in two ways. Either there is here a special type of anaerobic respiration which has nothing to do with alcoholic fermentation and takes place without the participation of the enzymes of fermentation, or else the newly formed alcohol or its forerunner is used as soon as it is formed and alcohol does not accumulate. The following table from Kostychev will illustrate the point:

Plant material	CO ₂ in mg.	Alcohol in mg.	CO ₂ C ₂ H ₅ OH
<i>Mucor racemosus</i>	696	688	100: 99
<i>Aspergillus niger</i>	530	500	100: 94
Flowers of <i>Acer platanoides</i>	736	786	100: 107
Roots of <i>Daucus carota</i>	318	324	100: 102
Sweet apples "Sinap".....	379	301	100: 80
Oranges.....	142	99	100: 70
Seedlings of <i>Lepidium sativum</i>	487	277	100: 57
Leaves of <i>Acer platanoides</i>	287	167	100: 58
Leaves of <i>Syringa vulgaris</i>	308	171	100: 56
Leaves of <i>Prunus padus</i>	456	232	100: 51
Roots of <i>Brassica rapa</i>	230	114	100: 49
Sour apples "Anton".....	277	117	100: 42
Potato tubers <i>Magnum bonum</i>	90	6	100: 7
Fruiting bodies of mushroom (<i>Psalliota campestris</i>)	1563	0	100: 0

The behavior of potatoes² and of mushrooms,³ which liberate considerable quantities of CO₂ but do not produce alcohol, is especially remarkable. The assumption is not precluded that in these cases the alcohol or the acetaldehyde is consumed by unknown secondary processes. However, this explanation does not hold for all cases. Thus sugar-free peptone cultures of *Aspergillus niger* do not produce the slightest amount of ethyl alcohol and also contain no zymase,⁴ since they are unable to ferment sugar that is added. Therefore this is a very special type of anaerobic respiration which will be considered later.

The chemical nature of anaerobic respiration which proceeds without forming alcohol is still unknown. As for the alcoholic fermentation of seed-plants, it is identical with that of yeasts, but in most seed-plants the rate of fermentation is

¹ Kostytschew, S. Ber. bot. Ges. 26a: 167. 1908; Zeitschr. f. physiol. Chem. 65: 350. 1910; Ber. bot. Ges. 31: 125. 1913; cf. also Palladin, W. und S. Kostytschew. Ibid. 25: 51. 1907; Zeitschr. f. physiol. Chem. 48: 214. 1906.

² Kostytschew, S. Ber. bot. Ges. 31: 127. 1913; cf. also Boysen-Jensen, P. Biol. medd. af det danske vidensk. selskab. 4: 1. 1923.

³ Kostytschew, S. Ber. bot. Ges. 25: 44. 1907; Zeitschr. f. physiol. Chem. 65: 350. 1910.

⁴ Kostytschew, S. und M. Afanassjewa. Jahrb. wiss. Bot. 60: 628. 1921.

lower. Hexose-phosphatase is very common in the plant kingdom.¹ Acetaldehyde has been captured during the anaerobic respiration of various fungi² by means of the sulphite method. Previously the idea was widespread that molecular hydrogen is formed during the anaerobic respiration of plants containing mannite, because the latter is said to split off hydrogen when it changes to sugar.³ This hypothesis proved to be erroneous;⁴ mannite is fermented only in the case of bacterial infection, whereby hydrogen is actually formed. This, however, is a process which has nothing to do with the physiological transformation in plants containing mannite.

26. THE GENETIC CONNECTION BETWEEN ANAEROBIC RESPIRATION AND OXYGEN RESPIRATION

Pflüger⁵ observed the anaerobic release of CO₂ in the case of frogs and on the basis of this observation expressed the fruitful thought that anaerobic respiration is neither a pathological phenomenon nor a biological adaptation but is the most important step in normal respiration. During anaerobic respiration there are formed, according to Pflüger's view, easily oxidized substances which are then attacked by atmospheric oxygen. Since the oxidizing enzymes were still unknown at that time, Pflüger was unable to define his theory more precisely.

Pflüger's theory was then extended to plant respiration by Pfeffer and Wortmann⁶ but because this process is very similar to fermentation by yeast the formation of alcohol must be taken into consideration. Above all, there is the question as to why alcohol does not accumulate in seed-plants with free access to air. Pfeffer assumed that alcohol is an intermediate product of respiration and is therefore totally consumed in the presence of air. Wortmann thought that he could show that alcohol is used in the synthesis of sugar. Eventually these hypotheses proved to be untenable; they were not based on experiments. Various other investigators claimed that anaerobic respiration is only a pathological phenomenon preceding death.⁷

At first the experimental investigation of anaerobic respiration did not produce results in favor of the theory of the genetic connection between anaerobic and normal respiration. The theories of Pfeffer and Wortmann were so phrased that they required a constant value of $\frac{I}{N}$ for all plants, but it was actually found that this value fluctuates within wide limits.⁸ On the other hand, the thorough investigations of Diakonow⁹ showed that molds have an anaerobic

¹ Nemec, A. und F. Duchon. *Biochem. Zeitschr.* 119: 73. 1921.

² Cohen, Clara. *Biochem. Zeitschr.* 112: 139. 1920; Neuberg, C. und C. Cohen. *Ibid.* 122: 204. 1921; Eliasberg, P. *Jour. landwirt. Inst. in Petersburg*, p. 74. 1921 (Russian).

³ Muntz. *Compt. rend.* 80: 178. 1875; *Ann. de chim. et de physique* (5) 8: 56. 1876; de Luca. *Ann. sci. nat.* (6) 6: 286. 1878.

⁴ Kostytschew, S. *Ber. bot. Ges.* 24: 436. 1906; 25: 178. 1907; *Physiol.-chemical investigations in plant respiration.* 1910 (Russian).

⁵ Pflüger. *Pflügers Arch. f. ges. Physiol.* 10: 251. 1875.

⁶ Pfeffer, W. *Landwirt. Jahrb.* 7: 805. 1878; Wortmann, J. *Arb. d. bot. Inst. Würzburg.* 2: 500. 1880.

⁷ Nägeli, C. *Theorie der Gärung.* 1879; Brefeld, O. *Landwirt. Jahrb.* 5: 281. 1876; Sachs, J. *Vorlesungen über Pflanzenphysiologie.* 1882; Borodin, J. *Bot. Zeit.* 39: 127. 1881; Godlewski, E. *Jahrb. wiss. Bot.* 13: 524. 1882; Reinke, J. *Bot. Zeit.* 41: 65. 1883.

⁸ Wilson. *Flora.* 65: 93. 1882; Möller. *Ber. bot. Ges.* 2: 306. 1884.

⁹ Diakonow, N. *Arch. slaves de biol.* 1: 531. 1886; 4: 31 and 121. 1887; *Ber. bot. Ges.* 4: 411. 1886. In a later paper, Diakonow claimed that anaerobic respiration could even be observed in the case of some molds grown on sugar solutions. Cf. Diakonow. *Arb. d. Naturforscherges. Petersburg.* 23: 1. 1893.

respiration only when fed with sugar; it ceases entirely if other organic substances are the source of carbon although these often make possible a good growth and a vigorous oxygen respiration. It was concluded from this that oxygen respiration takes place without the cooperation of anaerobic cleavage processes.

After Diakonow¹ and soon after that Palladin² had observed an increase in the anaerobic respiration of seed-plants when sugar was added, and had interpreted this according to Diakonow's theory, even Pfeffer found it necessary to give up his earlier theory.³ The views of the opponents of the theory of genetic relationship, especially Diakonow, Palladin and Godlewski, prevailed for a time.

However, at the beginning of the present century the theory of the genetic connection between anaerobic and aerobic respiration was restated in such a way that the fluctuations of the value of $\frac{I}{N}$ no longer counted against it.⁴ The first objection was thus removed. Soon the other important objection was gotten around; it became evident that Diakonow's experiments had been incorrectly interpreted and erroneous conclusions had resulted. Actually there is anaerobic respiration on all nutrient solutions on which the molds can develop.⁵ Later it was even found that non-sugars which are assimilated and which are free from nitrogen, such as tartaric acid, quinic acid, lactic acid, glycerine, mannite, etc., are converted by the fungi into sugar which is then fermented in the usual way.⁶ Palladin and Godlewski, two prominent opponents of the theory of genetic relationship, changed their views soon afterwards and produced many facts and arguments in favor of this theory. The theory has now won general recognition and only a few scattered voices are being raised against this conception.⁷

After the identity of the anaerobic respiration of most plants with alcoholic fermentation had been proved,⁸ it was possible to define better the relationship of anaerobic to normal respiration. The probable explanation is that the products of the anaerobic cleavage of sugar are burned by oxidizing enzymes. This hypothesis is supported by the formation of easily oxidized substances during temporary anaerobiosis. There is a tremendous increase in oxygen respiration after confinement for a time in a space containing no oxygen.⁹ Moreover,

¹ Diakonow, N. Arch. slaves de biol. 3: 6. 1887; Ber. bot. Ges. 4: 1. 1886.

² Palladin, W. Bull. Soc. Nat. de Moscou. 62: 44. 1886 (Russian); Rev. gén. bot. 6: 201. 1894.

³ Pfeffer, W. Untersuch. d. bot. Inst. Tübingen. 1: 105. 1881-1885; Lehrbuch der Pflanzenphysiologie, Vol. I. 1897.

⁴ Kostytschew, S. Jour. f. exp. Landwirt. 2: 580. 1901 (Russian); Jahrb. wiss. Bot. 40: 563. 1904; and others.

⁵ Kostytschew, S. *loc. cit.*; also Ber. bot. Ges. 20: 327. 1902; Investigations of the anaerobic respiration of plants. 1907 (Russian).

⁶ Kostytschew, S. Zeitschr. f. physiol. Chem. 111: 236. 1920; Kostytschew, S. und M. Afanassjewa. Jour. russ. bot. Ges. 2: 77. 1917 (Russian); Jahrb. wiss. Bot. 60: 628. 1921.

⁷ Polowzow, V. Investigations in plant respiration. 1901 (Russian); Maquenne, L. et E. Demoussy. Compt. rend. 123: 373. 1921; Boysen-Jensen, P. Biol. medd. af det danske vidensk. selskab. 4: 1. 1923.

⁸ Godlewski, E. und Polzeniusz. Bull. de l'acad. des sci. de Cracovie, p. 267. 1897; p. 227. 1901; Kostytschew, S. Ber. bot. Ges. 25: 44. 1907; Kostytschew and Afanassjewa. *loc. cit.*

⁹ Maquenne, L. Compt. rend. 119: 100 and 697. 1904; Palladin, W. Centralbl. f. Bakt. (II) 11: 146. 1904.

the alcoholic fermentation of yeast is much stimulated by extracts of plants and of muscles; on the other hand, the rate of respiration of animal tissue increases under the influence of yeast extract.¹ Moreover, the respiration of animal tissues requires the same hexose phosphate which plays a prominent rôle in alcoholic fermentation.² All this evidence supports the hypothesis that an increase in alcoholic fermentation, *i.e.*, in the first stage of the process of respiration, increases the rate of respiration.^x

Respiration is therefore chemically even more complicated than fermentation because the latter is only the first phase of the respiratory process. In addition to the processes of fermentation, respiration includes an oxidizing apparatus which burns the products of fermentation to CO₂ and water. It is now conceivable that seed-plants do not develop alcoholic fermentation to the extent required to support their entire operation but only to prepare material for the action of the oxidizing enzymes. We already know (p. 396) that for the same output of energy much less sugar is broken down in respiration than in alcoholic fermentation. Numerous results of various investigators show that the oxidizing enzymes of plants are unable to attack sugar directly. A previous treatment of sugar with yeast, however, makes possible the subsequent oxidation.

Now the question is, what products of fermentation are affected by oxidizing enzymes? Direct experiments have proved that ethyl alcohol is not an intermediate product of respiration.³ This result is easy to understand because ethyl alcohol as a rule is less liable to oxidation than sugar. On the contrary, fermented sugar solutions contain unidentified substances which are burned, with the formation of CO₂, by oxidizing enzymes of the seed-plants.⁴ On the basis of these results, the author of this book has proposed a theory which states that sugar is first attacked by the enzymes of fermentation but that the end-products of alcoholic fermentation are formed only in the case of a weak oxidizing activity, such as we find in yeast and in the *Mucoraceae* which ferment vigorously. In the case of typically aerobic plants, there are such large quantities of oxidizing enzymes present that the labile intermediate products of fermentation are oxidized before alcohol is formed. The relationship between fermentation and respiration can be shown by the following scheme:

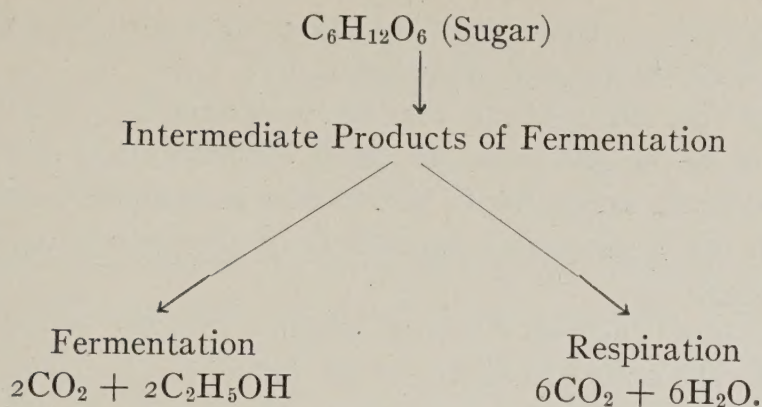
¹ Meyerhof, O. Pflügers Arch. f. ges. Physiol. 170: 267 and 428. 1918; Zeitschr. f. physiol. Chem. 101: 165. 1918; 102: 1. 1918.

² Embden, G. und Laqueur. Zeitschr. f. physiol. Chem. 93: 94. 1914; 98: 181. 1916; *cf.* also Embden, Griesbach und Schmitz. *Ibid.* 93: 124. 1914; Embden, Griesbach und Laqueur. *Ibid.* 93: 124. 1914.

^x Among the experimental investigations of this question which have not been mentioned elsewhere in this chapter, there have been extensive studies reported by Genevois who agrees that anaerobic respiration is like fermentation in the unicellular green algae. On this point see Genevois. Biochem. Zeitschr. 186: 461-473. 1927. For other papers by the same writer, see Rev. gén. bot. 40: 654 and 735. 1928, and 41: 154-184. 1929.—*Ed.*

³ Kostytschew, S. Biochem. Zeitschr. 15: 164. 1908; Jour. russ. bot. Ges. 1: 182. 1916 (Russian); Physiol.-chemical investigations in plant respiration. 1910 (Russian); Thomas, M. Biochem. Jour. 19: 927. 1925.

⁴ Kostytschew, S. *loc. cit.*; also Zeitschr. f. physiol. Chem. 67: 116. 1910. These experiments are the first instances of the oxidation of plant substances to inorganic products by means of plant enzymes outside of living cells.



This idea is supported by the recognition of acetaldehyde and pyruvic acid as intermediate products of alcoholic fermentation. If sugar should be respired by way of the intermediate stage of ethyl alcohol, we should have the following picture. Sugar is seized by the enzymes of fermentation and becomes alcohol by way of acetaldehyde. Later the alcohol is oxidized again by way of acetaldehyde because any other kind of oxidation of alcohol is hardly conceivable. In any case, such an unnecessary repetition of the formation of aldehyde is only barely possible.

So far we do not know what intermediate products of fermentation serve as normal intermediate products of respiration. Probably the oxidizing enzymes seize only the unsaturated radicals (p. 430) which are formed in the process of fermentation. The anaerobic respiration of animal tissue is, to all appearances, analogous not to alcoholic fermentation but to lactic acid fermentation.¹ Still it probably also precedes oxygen respiration and, like alcoholic fermentation and normal respiration, involves the formation of hexose phosphate. This shows that substances are oxidized in animal tissues which are formed from sugar even before the forking of the process of fermentation into alcoholic and lactic acid fermentation.

From the foregoing exposition it is clear that the respiration of sugar is closely connected with fermentation in the case of both plants and animals.

There still remains to be described now the oxidative end-stages of the processes of respiration. First however, it is necessary to learn about autoxidations in general and especially the oxidizing enzymes and their effects.

27. AUTOXIDATION AND OXIDIZING ENZYMES

It is acknowledged that Lavoisier was the first to prove that oxidations are due to the fixation of oxygen. Later investigations by various workers showed that autoxidations at the expense of molecular oxygen often introduce complicated processes, because the absorption of atmospheric oxygen by autoxidizable substances is not uncommonly accompanied by the oxidation of compounds which of themselves do not fix molecular oxygen at all or only very slowly. Thus phosphine is quickly oxidized by atmospheric oxygen. With it there easily occurs a simultaneous oxidation of indigo white which of itself proceeds

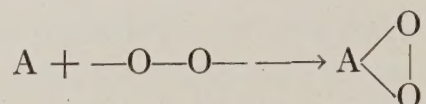
¹ Embden, G. und F. Kraus. *Biochem. Zeitschr.* 45: 1. 1912; Embden, Kalberloh und Engel. *Ibid.* 45: 45. 1912; Oppenheimer, S. *Ibid.* 45: 30. 1912; Kondo, K. *Ibid.* 45: 63 and 88. 1912.

slowly and incompletely. Analogous phenomena have also been observed for the autoxidation of aldehydes and other substances.

Thus it is clear that autoxidation does not lead directly to the formation of oxides as seemed to be the case from the classic experiments of Lavoisier. The processes of autoxidation are rather to be regarded as coupled reactions. After their investigation the physiological significance of chemical induction was first correctly understood.

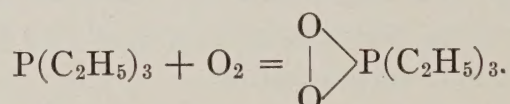
The basic idea of all theories of autoxidation is that the inactive molecular oxygen changes to the active form under the influence of various substances, and can then oxidize some other substances which are scarcely affected by molecular oxygen. Different authors do not represent the active form of oxygen in the same way. For example, Schönbein¹ assumes an intermediate formation of ozone while other workers suppose that hydrogen peroxide is formed² or that oxygen is ionized.³ At this point we can consider more fully only the theories of Bach-Engler, Warburg and Wieland.

Bach⁴ assumes that oxygen is active in the processes of autoxidation in the form of unsaturated molecules —O—O— ; thus there is always an addition of two atoms of oxygen to form the so-called moloxides which have the properties of peroxides:

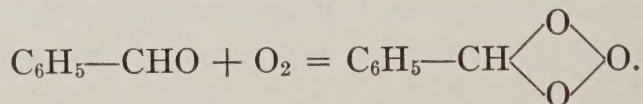


A. Engler⁵ also proposed this theory entirely independently of Bach.

It was actually proved by experiments that in the case of the autoxidation of triethyl phosphine $\text{P}(\text{C}_2\text{H}_5)_3$, for example, a moloxide is formed:⁶



Labile moloxides are also formed by the autoxidation of aldehydes. Thus benzaldehyde oxidizes as follows:⁷



¹ Schönbein, C. F. *Verhandl. d. Naturforscherges. Basel.* 3: 516. 1863; *Jour. f. prakt. Chem.* 105: 198. 1868; *Zeitschr. f. Biol.* 4: 367. 1868.

² Traube, M. *Ber. chem. Ges.* 15: 222, 659 and 2491. 1882; 16: 123 and 463. 1883; 18: 1877, 1887, 1890 and 1894. 1885; 19: 1111. 1886; 26: 1471. 1894; and others.

³ van't Hoff. *Zeitschr. f. physikal. Chem.* 16: 471. 1897; *Chem. Zeit.* 20: 807. 1896; *Jorissen. Ber. chem. Ges.* 30: 1951. 1897; and others.

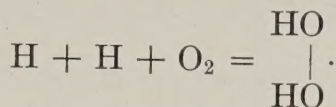
⁴ Bach, A. *Compt. rend.* 124: 951. 1897; *Moniteur scient.* (4) 11(II): 479. 1897; *Arch. des. sci. phys. et nat. Genève* (4) 35: 240. 1913.

⁵ Engler, A. und Wild. *Ber. chem. Ges.* 30: 1669. 1897; Engler. *Ibid.* 33: 1090 and 1109. 1900; Engler und Weissberg. *Ibid.* 31: 3055. 1898; 33: 1097. 1900; *Idem.* *Kritische Studien über die Vorgänge der Autoxydation.* 1904; Engler und Herzog. *Zeitschr. f. physiol. Chem.* 59: 327. 1909; Engler. *Zeitschr. f. Elektrochem.* 18: 945. 1912; and others.

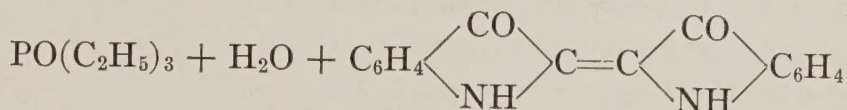
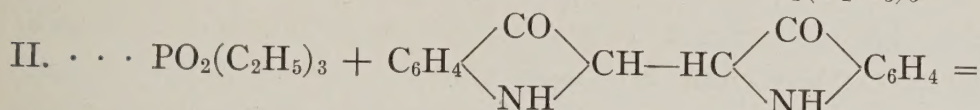
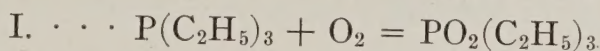
⁶ Engler und Weissberg. *Ber. chem. Ges.* 31: 3055. 1898.

⁷ Bach, A. *Moniteur scient.* (4) 11(II): 479. 1897; Luther und Schilow. *Zeitschr. f. physikal. Chem.* 46: 811. 1903.

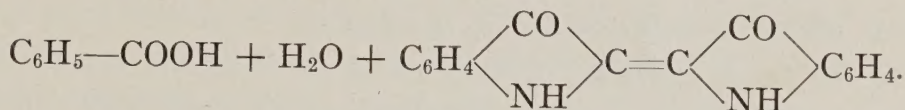
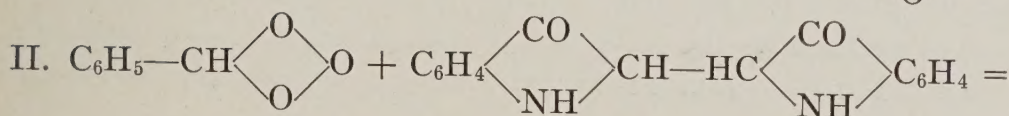
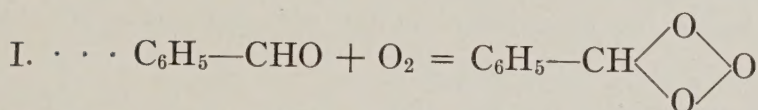
Even hydrogen is oxidized in the flame or under the influence of platinum black not first to water but to hydrogen peroxide:¹



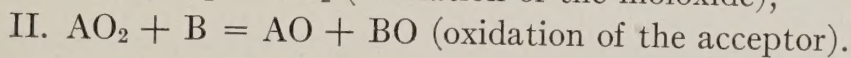
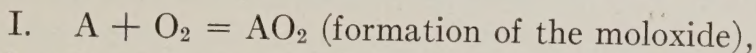
The moloxides formed by autoxidation are always more powerful agents of oxidation than molecular oxygen of the atmosphere. Consequently not only excess amounts of the autoxidator but also of other substances can be oxidized by moloxides; in this case, the velocities of the reactions concerned play the leading rôles. Thus the oxidation of indigo white by the autoxidation of triethyl phosphine or benzaldehyde is due to the following partial processes:



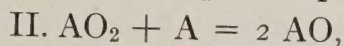
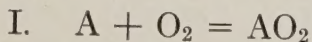
or



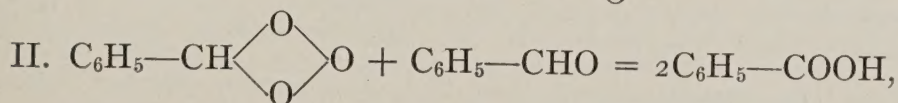
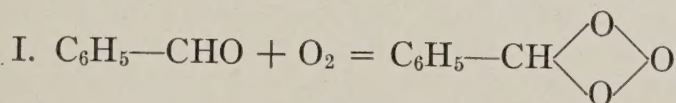
This is an example of the so-called coupled reactions. The substances which are easily oxidized by moloxides but are attacked with difficulty by atmospheric oxygen are called acceptors. The general scheme for coupled oxidation processes is therefore as follows:



If no special acceptor takes part in the reaction, the excess of the autoxidator acts as the acceptor and the entire process is represented as follows:

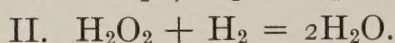
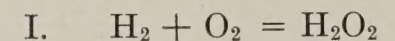


for example,

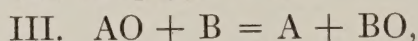
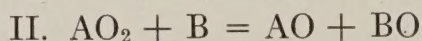
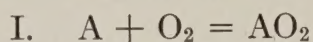


¹ Traube, M. Ber. chem. Ges. 18: 1897. 1885; 19: 1111. 1886; Engler, A. Ibid. 33: 1109. 1900.

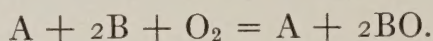
or



In a few special cases, the entire process of autoxidation is not called chemical induction but catalysis, in that the autoxidator passes on to the acceptor all of the oxygen taken from the air:

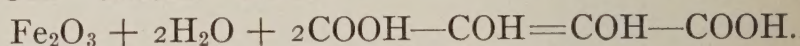
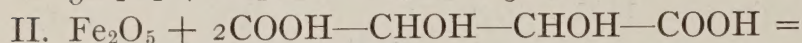
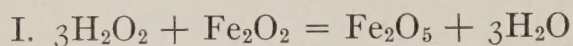


or taken together:



It is remarkable that the increase of the oxidation potential which is brought about in autoxidation by the formation of moloxides, can go even farther because there appears new peroxide-like substances with powerful oxidizing properties. Likewise in the living protoplasm there is often a chemical induction consisting of several stages.

For example, if we undertook to oxidize tartaric acid by atmospheric oxygen, we might first change the molecular oxygen into hydrogen peroxide by means of the induction processes just described. Hydrogen peroxide oxidizes indigo white but it does not attack tartaric acid. However, in the presence of a ferrous salt, tartaric acid is oxidized by the secondary ferrous peroxide Fe_2O_5 ,¹ since the oxidation potential of ferrous peroxide is higher than that of hydrogen peroxide:²



The red ferric peroxide Fe_2O_5 contains two atoms of active oxygen, after the removal of which it changes to brown ferric oxide Fe_2O_3 .

According to Bach and Chodat,³ physiological oxidation in plant cells is accomplished according to the same scheme which had been drawn up by Bach, Engler and their co-workers for the processes of slow oxidation *in vitro*. The so-called oxidizing enzymes show effects which resemble the processes of activation of molecular oxygen. *Laccase* from the Japanese lacquer tree was the first oxidizing enzyme to be prepared.⁴ It oxidizes various phenols and tannins and turns gum guaiacum to a deep blue color. Oxidizing enzymes were then isolated from the latex of various rubber trees⁵ and finally these enzymes proved

¹ Fenton. Jour. Chem. Soc. (London) 65: 899. 1895.

² Manchot und Wilhelms. Ber. chem. Ges. 34: 2479. 1901; Luther und Schilow. Zeitschr. f. physikal. Chem. 46: 777. 1903.

³ Bach und Chodat. Ber. chem. Ges. 35: 1275, 2466 and 3943. 1902; 36: 600 and 606. 1903; Idem. Arch. des. sci. phys. et nat. Genève. 17: 477. 1904; and others.

⁴ Yoshida. Jour. Chem. Soc. (London) 43: 472. 1883; Bertrand, G. Compt. rend. 118: 1215. 1894; 120: 266. 1895; 121: 166. 1895; 122: 1132. 1896; 145: 340. 1907; Idem. Bull. Soc. Chim. de France (4) 1: 1120. 1907; Hérissé et Doby. Jour. de pharm. et de chim. 30: 289. 1909.

⁵ Spence, D. Biochem. Jour. 3: 165 and 351. 1908; Cayla, V. Bull. Soc. Biol. 65: 128. 1908; and others.

to be generally widespread substances.¹ It is not easy to distinguish individual enzymes by their effects, because enzymatic oxidations outside of living cells are accompanied by condensation and resining and therefore by processes which certainly do not take place in living cells. In addition to laccase, oenoxidase from grapes² which oxidizes tannins, and tyrosinase³ which oxidizes tyrosine, have been described as separate enzymes but strong proofs are lacking for the specificity of these enzymes. Various color reactions, such as a blue coloration with gum guaiacum, a brown color with guaiacol, a red color with aloins, etc. have been proposed as tests for oxidizing enzymes. However, all these tests are unreliable; they may give positive results not only by autoxidations but also by the transfer of atomic oxygen, while oxidizing enzymes are of interest only as autoxidators. Consequently the proof of the absorption of oxygen from the surrounding air is the only reliable test of oxidizing enzymes.⁴

According to the theory of Bach and Chodat, all oxidizing enzymes consist of two components—an organic peroxide, the so-called oxygenase, and an enzyme which raises the oxidation potential of peroxides and is called peroxidase.⁵ Like ferrous salts, peroxidase activates not only the organic peroxides isolated from plants but also hydrogen peroxide. It is not improbable that the mechanism of peroxidase action is analogous to that of the action of the ferrous ion and is based on the formation of a secondary peroxidase peroxide. In this connection it should be remembered that preparations of oxidizing enzymes usually contain considerable quantities of iron or manganese.⁶ Since the presence of a metal is not essential, it is evident that various organic peroxides exert very powerful oxidising effects and are not inferior to iron or manganese peroxides as a means of oxidation. From the standpoint of the Bach-Engler theory, the general mechanism of physiological oxidation processes consists in the fixation of atmospheric oxygen by specific autoxidators, the so-called oxidases,⁷ whereby the first products are organic peroxides or oxygenases. Under certain conditions, there may be a direct oxidation of different substances by means of oxygenases but for the most part their oxidation

¹ Literature on oxidizing enzymes up to 1905 by **Porodko**. *Beih. z. bot. Centralbl.* 16: 1. 1904; more recent literature by **Begemann**, O. *Zeitschr. f. allgem. Physiol.* 16: 603. 1914; **Idem**. *Pflügers Arch. f. ges. Physiol.* 161: 45. 1915; [also complete account of oxidases as understood in 1909 by **Kastle**, J. H. The oxidases and other oxygen-catalysts concerned in biological oxidations. U. S. Treas. Dept. Hyg. Lab. Bull. 59 which also contains a valuable bibliography of 467 entries].

² **Cazeneuve**. *Compt. rend.* 124: 406 and 781. 1907; **Bouffard**. *Ibid.* 124: 706. 1907; **Laborde**. *Ibid.* 126: 536. 1898.

³ **Bourquelot**, E. et **G. Bertrand**. *Jour. de pharm. et de chim.* (6) 3: 177. 1896; **Idem**. *Bull. soc. mycol.*, p. 18 and 27. 1896; p. 65. 1897; **Gessard**. *Compt. rend. soc. de biol.* (10) 5: 1033. 1898; **Beijerinck**. *Verslagen d. Afdeeling Natuurkunde, Kgl. Akad. Wiss. Amsterdam*, p. 932. 1912; **Idem**. *Chem. Weekbl.* 16: 1894. 1919; **Krainsky**. *Centralbl. f. Bakt.* (II) 41: 669. 1914; **Bertrand**, G. *Compt. rend.* 122: 1215. 1896; 123: 463. 1896; **Chodat et Staub**. *Arch. des sci. phys. et nat.* (4) 23: 265. 1907; **Bach**, A. *Ber. chem. Ges.* 39: 2126. 1906; **Bertrand**, G. et **Mutermilch**. *Compt. rend.* 144: 1285. 1907; **Idem**. *Ann. Inst. Pasteur.* 21: 833. 1907; **Zerban**, F. W. *Jour. Ind. Eng. Chem.* 10: 814. 1918; and others.

⁴ **Kostytschew**, S. *Physiol.-chemical investigations in plant respiration.* 1910 (Russian).

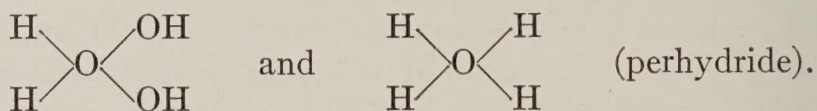
⁵ **Bach**, A. *Ber. chem. Ges.* 37: 3787. 1904; 38: 1878. 1905; 40: 3185. 1907; **Wolff**. *Contribution à la connaissance de divers phénomènes oxydasiques naturels et artificiels.* 1910.

⁶ **Bertrand**, G. *Ann. de chim. et de physique* (7) 12: 115. 1897; *Compt. rend.* 124: 1032 and 1355. 1897; **Sarthou**. *Jour. de pharm. et de chim.* (6) 11: 583. 1900; 13: 464. 1902; and others.

⁷ **Kostytschew**, S. *loc. cit.*

potential is raised by the formation of secondary peroxides through the action of peroxidase. The assumption is not precluded that in some cases there is a repeated increase of the oxidation potential whereat new peroxides are formed.¹ Of all the foregoing substances, only the peroxidases can be regarded as enzymes. By analogy with slow oxidations in the field of general chemistry, we are justified in supposing that in many cases the rest of the process is not catalysis but induction. It is therefore quite probable that coupled reactions, the importance of which in physiological processes has not been sufficiently appreciated, often play a leading rôle in biological oxidations. Euler and Bolin² determined the chemical composition of laccase from *Medicago sativa* and found that it is only a mixture of salts of some organic acids. Among the acids present in large quantities were glycolic acid $\text{CH}_2\text{OH}-\text{COOH}$, glyoxylic acid $\text{CHO}-\text{COOH}$ and mesoxalic acid $\text{COOH}-\text{CO}-\text{COOH}$.

It was pointed out above that Bach³ also represents the mechanism of biological reductions in the same way as that of oxidations. He believes that the enzyme perhydridase and substances with active hydrogen are functional in all reductions. Perhydridase is said to activate the hydrogen in the same way that peroxidase raises the oxidation potential of peroxides. Bach considers water to be an unsaturated compound of quadrivalent oxygen and assumes that the free valences of oxygen become saturated by the ions of the dissociated water:



Perhydride is activated by perhydridase and can thereby reduce various substances by means of its surplus hydrogen.

O. Warburg⁴ has proposed a remarkable physical-chemical theory of cell respiration which is provisionally supported only by experiments with animal cells and inorganic models, and yet with some additions it might be applicable to plant respiration. From a series of experiments on the cells of the blood of birds and on sea urchin eggs on the one hand, as well as on various charcoal powders on the other, Warburg concludes that "cell respiration is a capillary-chemical process which takes place at the iron-containing surfaces of the *fixed* cell constituents." That is, it was found that various amino acids are burned to NH_3 , CO_2 and H_2O when adsorbed to charcoal and that the velocities and accelerations of the oxidation processes are thereby exactly the same as in the case of true cell respiration. The experiments on the effect of various narcotics are especially convincing. For equally strong checking of respiration this effect can be expressed by the following equation:

$$x \cdot F = K,$$

¹ According to the latest information, some plants also form ozone. Cf. Coupin, H. Compt. rend. 178: 1572. 1924.

² Euler, H. v. und J. Bolin. Zeitschr. f. physiol. Chem. 61: 1. 1909.

³ Bach, A. Ber. chem. Ges. 42: 4463. 1909; Idem. Handb. d. Biochem. (by Oppenheimer) Erg.-Bd., p. 151. 1913.

⁴ Warburg, O. Biochem. Zeitschr. 119: 134. 1921; 136: 266. 1923; 142: 518. 1923; Zeitschr. f. Elektrochem., p. 70. 1922; [Science. 61: 575-582. 1925].

where x represents the number of adsorbed molecules of the narcotic and F is the area occupied by each molecule on the surface of the adsorbent. In this way it appears that the chemical nature of the various narcotics is of no importance and the retardation of oxidation is due only to the fact that the substances to be oxidized, *e.g.*, the amino acids, are not oxidized in the solution. The following table from Warburg shows that $x \cdot F$ actually varies but a little for equally strong retardation and is therefore almost a constant.¹

Substance	K
Dimethylurea.....	9.0
Diethylurea.....	6.9
Phenylurea.....	8.7
Acetamide.....	7.3
Valeramide.....	6.9
Acetone.....	8.3
Methylphenylketone.....	8.0
Amyl alcohol.....	7.9
Acetonitrile.....	7.7

Since oxidation is checked by such small amounts of hydrocyanic acid, it alone occupies a special position inasmuch as practically no noticeable displacement of the substance to be oxidized could be effected. However, it is evident that hydrocyanic acid unites with ferric ions and consequently this causes a displacement of respiratory material from the regions of oxidation, since ferric ions are said to be indispensable for the oxidation.

It is certainly very welcome to have physiological-chemical theories of respiration finally arrive. Without doubt a thorough explanation of various processes which are physiologically important processes can be obtained only by drawing on the capillary phenomena. Warburg himself² also expresses the opinion "that the conception of respiration as a surface reaction will point the way followed by the transformation of energy in the living cell," since the forces of surface tension perform work. Moreover, the assumption is not precluded that various supposed "enzyme effects" might often be traced to the fact that the particular process represents a boundary surface phenomenon by which the concentration of the substances concerned in the reaction is uncommonly high.³ However, in the field of plant respiration some facts have not yet been harmonized with various points of the Warburg theory as it now stands. Thus ferric ions for the most part are not detectable in plant cells because the iron exists in rather stable organic combination. On the other hand, it would be premature to deny the participation of peroxidases in the respiratory processes. According to Willstätter, these enzymes are found to be

¹ A constant which is proportional to the magnitude of $x \cdot F$.

² Warburg, O. *loc. cit.*

³ In this case the specific nature of the adsorbent is of secondary importance. Hence, an assumption of specific catalysts seems to be superfluous in many cases.

free from iron.¹ It is to be emphasized especially that sugars serve as the respiratory material of plants. A repetition of the Warburg model experiment by Meyerhof² shows that various amino acids but no sugars are burned under these conditions. It would certainly be most interesting to test fermented sugar solutions on the Warburg model. Meyerhof declares that hexose phosphate is attacked. From the standpoint of the theory of genetic relationship of the anaerobic with normal respiration, the results obtained by Meyerhof certainly do not contradict Warburg's theory of respiration. They could be used in favor of the assumption that a primary anaerobic cleavage of sugar is necessary for sugar respiration.³

An entirely different theory of the oxidation processes has been developed by Wieland.⁴ According to his view, the oxygen of water is utilized for all biological oxidations. The atmospheric oxygen only serves to fix the hydrogen of the water. In general, Wieland supposes that oxidations are always accompanied by simultaneous reductions, for always the hydrogen and not the molecular oxygen is said to be activated. The latter is said to remain entirely passive and to form no unsaturated molecules. According to Wieland, oxidation is due solely to the formation of active atomic hydrogen. If the active hydrogen of the water is tied up by a hydrogen acceptor, the residual unsaturated oxygen of the water can unite with various substances. Either atmospheric oxygen or various other substances could serve as hydrogen acceptors. In the latter case it is possible to carry out various oxidations and reductions with air excluded. Thus Wieland has proved that acetic acid fermentation, commonly thought to be an oxidation of ethyl alcohol by atmospheric oxygen, proceeds smoothly in the presence of methylene blue or meta-dinitro-benzene with oxygen entirely excluded. According to Wieland, a state of equilibrium is therefore precluded under these conditions because the active hydrogen is permanently fixed. As for the manner of action of peroxidases, Wieland assumes that these enzymes only activate the hydrogen of the polyphenols and make possible their oxidation by means of atmospheric oxygen. In fact it must be pointed out that the peroxidase preparations so far exhibited from plants only oxidize the hydrogen of phenols and are unable to break carbon

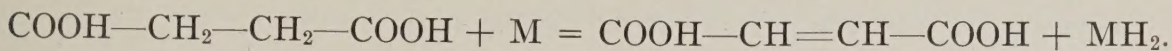
¹ Hence it is apparent that a biological activation of oxygen may also occur without the assistance of ferric ions. [However, the work of Willstätter and Pollinger (Liebigs Ann. d. Chem. 430: 269-319. 1923) showed that all active preparations of peroxidase do contain a trace of iron which is an impurity rather than a part of the peroxidase.]

² Meyerhof, O. und H. Weber. Biochem. Zeitschr. 135: 558. 1923.

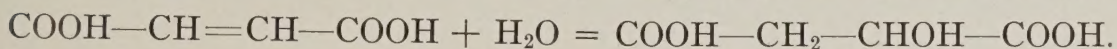
³ The latest discovery of Warburg and Yabusoe, according to which fructose undergoes oxidation in the presence of phosphates, is interesting. Cf. Warburg, O. und M. Yabusoe. Biochem. Zeitschr. 146: 380. 1924; Spoehr, H. A. Jour. Amer. Chem. Soc. 46: 1494. 1924. However, this oxidation is so feeble that it can not serve as the basis of normal respiration. On this point see Meyerhof, O. und K. Matsuoka. Biochem. Zeitschr. 150: 1. 1924. [The latter have confirmed and augmented the work of Warburg and Yabusoe. The presence of traces of iron in a certain form is said to be responsible for the slow but regular oxidation of fructose (but not glucose) at 37° and in pure oxygen. These experimental conditions and the absence of any effect on glucose weakens the argument for the general application of Warburg's suggestion. It is also true that a careful demonstration of the catalytic action of iron for the oxidation of fructose has been made only with alkaline solutions, whereas fructose is oxidized in neutral solutions of phosphate in Warburg's experiments (cf. Wind, F. Biochem. Zeitschr. 159: 58-67. 1925). It would seem that too little consideration was given to the possible catalytic effect of the phosphate.]

⁴ Wieland, H. Ber. chem. Ges. 45: 2606. 1912; 46: 3327. 1913; 47: 2085. 1914; 55: 3639. 1922.

chains and in general to unite the oxygen of peroxides with carbon. Wieland's theory may be successfully applied to cases in which the oxidation is wholly or partly due to the removal of hydrogen. Then it serves well and provides the explanation of processes which present difficulties for the Bach-Engler theory. An example of these is the following oxidation described by Thunberg.¹ Succinic acid is easily changed to fumaric acid in the presence of muscle extract and methylene blue (M) as the hydrogen acceptor:

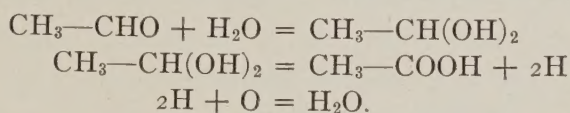


Furthermore, fumaric acid can change to malic by the fixation of water.

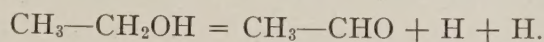


In this way it is possible to explain a whole series of biochemical reactions which involve a transfer of hydrogen or oxygen. It must be borne in mind, however, that there are objections to Wieland's theory as an explanation of all the processes of oxidation in plant cells.² Wieland³ himself expresses the opinion that his theory is generally valid and that the Bach-Engler theory should be abandoned as superceded.

It must be taken into consideration that two *experimentally established* important facts favor the Bach-Engler theory: I. The formation of moloxides by the autoxidation of some organic substances and, II. The activation of hydrogen peroxide and various other peroxides by means of peroxidases. The Wieland theory can explain some cases of *oxygen fixation* only questionably. For example, Wieland seeks to explain the autoxidation of aldehyde to carboxylic acids at the expense of the oxygen of water by means of the removal of water from the aldehyde hydrates:



Wieland bases this explanation on the fact that acetic acid bacteria oxidize ethyl alcohol to acetic acid in the absence of oxygen by way of the intermediate stage of acetaldehyde if the active hydrogen which is split off is taken up by methylene blue. The first stage (oxidation of ethyl alcohol to acetaldehyde), in the absence of oxygen, can be made intelligible only through an activation of hydrogen:



The second stage (oxidation of acetaldehyde to acetic acid) is also, according to Wieland, due to the fact that there is first a formation of aldehyde hydrate which then, owing to the activation of hydrogen, splits off atomic hydrogen and changes to acetic acid. Since no atmospheric oxygen could be sympathetically affected under his experimental conditions, Wieland claims that his explanation of the formation of acetic acid is the only one possible (*loc. cit.*). The reply to this is that in some cases aldehyde peroxides have been isolated in the case of the oxidation of this substance. To all appearances entirely different processes take place with oxygen excluded than with access to oxygen: aldehydes change to alcohols and acids according to the Cannizzaro reaction.

¹ Thunberg. Skand. Arch. f. Physiol. 30: 285. 1913; [40: 1. 1920].

² Bach und Michlin. Ber. chem. Ges. 60: 827. 1927; Bach und Nikolajew. Biochem. Zeitschr. 169: 105. 1926.

³ Wieland, H. Ber. chem. Ges. 55: 3639. 1922.

The foregoing objections do not have the effect of setting aside the Wieland theory. They only go to show that in some cases the Bach-Engler theory deserves the preference. In the case of biological oxidation processes there is probably hydrogen activation as well as oxygen activation. In some cases the oxidation proceeds according to the Bach-Engler scheme, but in other cases according to that of Wieland.

Another very interesting theory of the shifting of hydrogen in the molecule is based on the views of J. J. Thomson¹ and was developed by Quastel.²

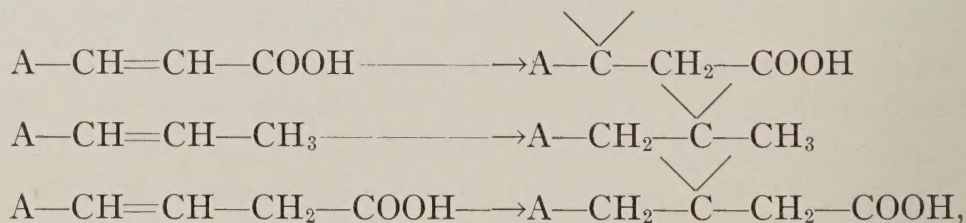
In the case of double bond between two carbon atoms $>C_1=C_2<$ (the simplest model), the two carbon atoms are joined by 4 electrons. An external electrical field can produce a shift of 2 electrons in a given direction. If the shifting of the 2 electrons goes so far that they are left attached to C_2 only, the double bond disappears and C_2 becomes saturated while C_1 is given free valences and is thereby activated. Without going further into the matter, it is clear that hydrogen atoms (protons) can be displaced in the same way as electrons. In this way the grouping $-CH=CH-$ changes to $-C-CH_2-$, $-CH=O$ to

$-C-OH$, etc. The electrical effect of such a shift of one hydrogen atom can

be produced by the origin of an electrical doublet on the hydrogen atom. If a system requires the addition of one electron for its normal saturation, the positive end of the doublet places itself against the molecule in which the given system is included. The opposite position is assumed in respect to a system which involves a superfluous electron. The following table will serve to explain this rule:

System	Electron No.	Surplus or Deficiency of Electrons	Effect on the α -C Atom	Polarity
$-CH_3$	7	+1	Repulsed from + electrode	Not polar
$-OH$	7	+1	Repulsed from + electrode	Polar
$-NH_2$	7	+1	Repulsed from + electrode	Polar
$-CH_2-COOH$	23	+1	Repulsed from + electrode	Polar
$-COOH$	17	-1	Drawn to + electrode	Polar
$-CONH_2$	17	-1	Drawn to + electrode	Polar
$-COCH_3$	17	-1	Drawn to + electrode	Polar

Accordingly different systems must exhibit unequal forms of activation, as e.g.,



assuming that A has no influence on the movement of the proton.

Analogous effects are also found in saturated molecules but only in the presence of hydrogen acceptors.

¹ Thomson, J. J. Phil. Mag. 46: 506. 1923; Nature. 112: 26. 1923.

² Quastel. Biochem. Jour. 20: 166. 1926; Réunion Inter. de Chimie Physique. Oct., 1928.

The advantage of this scheme over that of Wieland is that the shifting of the hydrogen can be explained and even predicted. For example, on the basis of the Quastel-Thomson theory the following facts can easily be explained:

1. The very widespread β -oxidation,
2. Asymmetrical oxidation of double bonds,
3. The α -oxidation of propionic acid,

and various other facts. This theory may also be of service in the interpretation of the processes of fermentation.

28. THE SUM TOTAL OF THE CHEMICAL PROCESSES IN PLANT RESPIRATION

We must collect all the foregoing facts and theories in order to obtain a correct conception of the chemical nature of plant respiration. First of all, it is important to decide which substances serve as material for respiration. On the basis of modern conceptions, the fermentable sugars are the normal materials. Boussingault¹ proved that carbohydrates in particular disappear during the respiration of seedlings. An addition of sugar always accelerates the rate of respiration of living cells² and in a few organs which respire especially vigorously, an enormous consumption of sugar can be detected by direct analysis.³ The proteins were once considered to be the especially active constituent of living protoplasm and it appeared to be unlikely that such an important vital process as respiration could take place without the participation of proteins. Hence it was assumed that only the proteins of the protoplasm are oxidized directly. Carbohydrates were required only to regenerate the proteins destroyed by oxidation.⁴ However, it is now known that proteins are less easily oxidized than sugar, and that sugar is burned at low temperatures through induction of oxidative processes. Therefore the old idea of a protein cleavage during normal respiration is to be regarded as obsolete. It is unanimously claimed to-day that the material for respiration is free molecules of sugar which form no part of the protoplasmic framework.

In the seed-plants almost all living cells are provided with a supply of sugars or other carbohydrates. All the polysaccharides such as starch, hemicelluloses and inulin, as well as glucosides, break down easily in the protoplasm through the action of specific enzymes to form simple sugars, particularly glucose and fructose, which as direct respiratory material undergo an oxidative cleavage.

Sugars and polysaccharides are also contained in large amounts in various lower plants. Starch is often replaced by glycogen in the higher fungi while among the disaccharides trehalose is especially common in the fungi. There is never lacking in these plants a supply of respiratory material which furnishes fermentable hexoses by means of hydrolytic cleavage.

¹ Boussingault. *Agronomie, chimie agric. et physiol.* 4: 245. 1868.

² Borodin, J. *Physiological investigations of the respiration of leafy shoots.* 1876 (Russian); Palladin, W. *Rev. g n. bot.* 5: 449. 1893.

³ Kraus, G. *Ann. du jardin bot. de Buitenzorg.* 13: 217. 1896; and others.

⁴ Detmer. *Jahrb. wiss. Bot.* 12: 287. 1879; *Ber. bot. Ges.* 10: 201 and 442. 1892; *Lehrbuch der Pflanzenphysiologie*, p. 153. 1893.

In the plants without chlorophyll, as well as in those which have it, the respiratory material is often deposited in the form of fat. The conversion of fat to sugar is a process which has not yet been done artificially but it proceeds easily in living plant cells.¹ The respiration of fat involves a comparatively low value² of $\frac{\text{CO}_2}{\text{O}_2}$ and undoubtedly is effected by way of the intermediate stage of sugar.

Various lower plants can live well and respire intensively at the expense of various kinds of organic substances. However, it has already been shown (p. 180 ff.) that organic foods containing no nitrogen are converted to sugar by the omnivorous molds.³ The chemical side of these transformations is still unknown but there is no doubt that the use in respiration of the sugar formed from different organic substances proceeds in exactly the same way as in the case of normal nutrition with sugar.

However, there seems to be still another kind of plant respiration which appears especially in starving cells. It is the respiration at the expense of the protoplasmic proteins. It is to be emphasized expressly that this respiration is not a normal type and takes place only in exceptional cases. It has been known for a long time that nucleoproteids decompose with prolonged starvation and there is a destruction of the protoplasmic framework.⁴ Of course, this respiration at the expense of the protoplasm has absolutely nothing to do with the hypothetical respiration which was assumed by old theories. Those theories spoke of a dissociation and oxidation of the protoplasmic proteins in the very cases in which a direct analysis proves the consumption of sugar alone. Actually there is a use of protein in respiration merely with a deficiency of sugar, as in etiolated bean leaves.⁵ This respiration is always considerably weaker than normal sugar respiration. Exhaustive experiments with molds show that so long as these organisms respire at the expense of sugar, no protein cleavage or working-up of amino acids can be noted in them. These processes are started only by starvation in the absence of sugar.⁶

Molds are also valuable experimental material in other respects; these omnivorous organisms get along well with peptone as the only source of carbon. Such peptone cultures make it possible to study protein respiration without starving the organism. In the absence of oxygen, neither sugar nor alcohol is formed in peptone cultures.

Since peptone cultures of *Aspergillus niger* liberate CO₂ in the absence of oxygen but form neither sugar nor alcohol, we may suppose that the oxygen

¹ Leclerc du Sablon. Compt. rend. 117: 524. 1893; 119: 610. 1894; Maquenne, L. Ibid. 127: 625. 1908. For the respiration of fat in the lower fungi see Flieg, O. Jahrb. wiss. Bot. 61: 24. 1922.

² Godlewski, E. Jahrb. wiss. Bot. 13: 524. 1882.

³ Kostytschew, S. Zeitschr. f. physiol. Chem. 111: 236. 1920; Kostytschew and Afanassjewa. Jour. russ. bot. Ges. 2: 77. 1917 (Russian); Jahrb. wiss. Bot. 60: 628. 1921.

⁴ See Zaleski, W. The transformation and rôle of the phosphorus compounds in the plant. 1912 (Russian).

⁵ Palladin, W. Rev. gén. bot. 5: 449. 1893.

⁶ Butkewitsch, W. Jahrb. wiss. Bot. 38: 147. 1902; Idem. Anniv. vol. for K. A. Timiriazeff, p. 495. 1916 (Russian); Kostytschew, S. und E. Tswetkova. Zeitschr. f. physiol. Chem. 111: 171. 1920.

respiration of these cultures is peculiar and differs in principle from the ordinary sugar respiration. On the strength of these results, Kostychev assumes that there are two kinds of plant respiration—"sugar respiration" and "protein respiration." The former is the usual, common type and proceeds by means of the participation of the enzymes of alcoholic fermentation. The latter is only initiated after stoppage of the supply of carbohydrates and takes place without manifestation of the enzymes of fermentation. The chemical side of the material transformations which proceed thereby is at present wholly unknown.

Sugar respiration has been studied somewhat better although there are also large gaps in this field. The changes in the sugar fall into two large stages—the primary anaerobic cleavage of sugar by enzymes of fermentation and the secondary oxidation of the cleavage products. The oxidation potential of the oxidizing enzymes of plants is much too low for a direct oxidation of sugar and in general to break carbon chains. The oxidizing enzymes so far investigated are able to unite with oxygen only the hydrogen of the polyphenols and other aromatic compounds. Carbon cannot be oxidized by a direct action of these enzymes. On the contrary, the author of this book has proved¹ that peroxidase isolated from wheat seedlings oxidizes *fermented* sugar solutions to form an abundance of CO₂ in the presence of hydrogen peroxide. The same peroxidase is unable to split pure glucose and form CO₂ by means of hydrogen peroxide. These results show that the oxidizing factors isolated from plants can burn plant compounds to CO₂ and that the intermediate products of fermentation in particular can function as material for respiration.²

Among these products, both acetaldehyde and pyroracemic acid may perhaps be oxidized. Still we know that chiefly glyoxal CHO—CHO is formed by the action of nitric acid on acetaldehyde. Glyoxal could easily be burned to CO₂ and H₂O by way of glyoxylic acid CHO—COOH or oxalic acid COOH—COOH. Pyroracemic acid could also be oxidized in an analogous way. Still it seems to be a more plausible hypothesis that the unsaturated substances formed during fermentation are the normal intermediate products of oxygen respiration.

According to the ratio of the relative velocities of the primary cleavage and the subsequent oxidation, the energizing process *in toto* can proceed somewhat differently, as shown by the following possibilities.

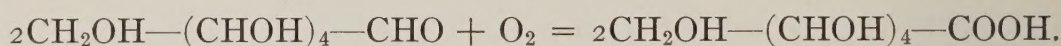
First Case. The ability of the plant to ferment is extremely great in comparison with the quantity of the oxidizing enzymes. For that reason the velocity of the primary cleavage of sugar is greater than that of the subsequent oxidation. Consequently, part of the sugar attacked by fermentation cannot be entirely consumed, even with free access to oxygen. The labile, intermediate products of the alcoholic fermentation are changed, under aerobic conditions, into the stable form of alcohol and CO₂, since the activity of the oxidizing enzymes does not suffice to effect a complete oxidation of all the products of fermentation.

¹ Kostytschew, S. Zeitschr. f. physiol. Chem. 67: 116. 1910.

² Kostytschew, S. Biochem. Zeitschr. 15: 164. 1908.

Second Case. The quantities of oxidizing enzymes and the fermentation capacity are so coordinated in the plant that the velocity of the primary sugar cleavage is always less than that of the subsequent oxidation. Consequently alcohol is never formed when air is admitted, since all the intermediate products pass over into the end-products of oxygen respiration. This is the usual respiration of the seed-plants and the strongly aerobic microorganisms in which there is a complete combustion of sugar even with low oxygen tension.¹ The following observation can serve to illustrate the unlike behavior of strongly aerobic fungi, such as *Aspergillus niger* on one hand, and the aerobic fermenting fungi such as yeast and *Mucors* on the other hand, in the case of a somewhat restricted access to oxygen. If *Aspergillus niger* is immersed in a sugar solution, it does not produce a trace of alcohol provided the surface of the solution is left in equilibrium with the air. The oxidizing action of this fungus is so strong that even the small amount of oxygen dissolved in the solution effects a complete oxidation of the respiratory material.² The fermenting yeasts and *Mucors* behave quite differently. Under the same experimental conditions they give rise to a vigorous alcoholic fermentation and the combustion of sugar is discontinued.

Third Case. The plant is a weak producer of fermentation; hence, the oxidizing enzymes are not only able to oxidize completely all the intermediate products of fermentation but they also attack the unchanged sugar, since their working capacity is not spent on the insignificant quantities of the fermentation products. An incompletely proved example of this case is that of the formation of gluconic acid by *Aspergillus niger*. M. Molliard³ has found that with a deficiency of mineral nutrients *Aspergillus niger* oxidizes grape sugar to gluconic acid:



It is characteristic of oxidizing enzymes of plants that they are able neither to break carbon chains nor to unite carbon atoms and oxygen. This has been discussed in previous statements.

The even combustion of the labile intermediate products of fermentation can, as we have already seen, be brought about either by the activation of oxygen or by the activation of hydrogen. The hypothesis is not precluded that the peroxidases, as well as other inductors of the oxidation processes in living plant cells, gradually raise the oxidation potential so much that not only is all the hydrogen of the respiratory material oxidized to water but all the carbon is also burned to CO_2 . This simple hypothesis is not impossible but other more plausible theories also deserve attention. Thus Palladin⁴ has proposed an

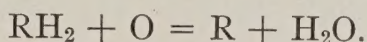
¹ Stich. Flora. 74: 1. 1891; Amm. Jahrb. wiss. Bot. 25: 1. 1893; Johannsen. Untersuch. d. bot. Inst. Tübingen. 1: 716. 1885.

² Kostytshew, S. and M. Afanassjewa. Jour. russ. bot. Ges. 2: 77. 1917 (Russian).

³ Molliard, M. Compt. rend. 174: 881. 1922; 178: 41. 1924.

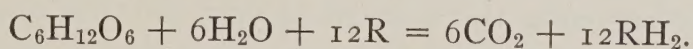
⁴ Palladin, W. Ber. bot. Ges. 26a: 125, 378 and 389. 1908; 27: 101. 1909; 29: 472. 1911; 30: 104. 1912; Zeitschr. f. physiol. Chem. 55: 207. 1908; Rev. gén. bot. 23: 225. 1911; Zeitschr. f. Gärungsphysiol. 1: 91. 1912; Palladin und Lwow. Ibid. 2: 326. 1913; Palladin and Z. Tolstoi. Mitt. d. Akad. d. Wiss. Petersburg, p. 93. 1913 (Russian).

interesting theory to explain the oxidation of the intermediate products of fermentation. This theory represents a combination of those of Bach-Engler and Wieland. According to Palladin, the so-called respiratory chromogens play a very important rôle in the respiratory process. Palladin applied the term respiratory chromogen to various substances which by autoxidation split off hydrogen and change to the corresponding pigment. For example, hydroquinone is oxidized to red quinone by peroxidase and indigo white to blue indigo. All such oxidations are due to a removal of two atoms of hydrogen with the formation of water:



According to Palladin, reserve respiratory chromogens are stored in the form of prochromogens. Under this term Palladin included many tannins, glucosides and similar compounds which liberate oxidizable chromogens on hydrolysis.

The respiratory pigments R which are formed from chromogens by oxidation are effective as hydrogen acceptors according to Palladin, while the oxidation of carbon is said by Wieland to take place by means of the oxygen of water. In living tissues the respiratory process, according to Palladin, is initiated by the catalytic dissociation of water. The oxygen of the water oxidizes the carbon of the intermediate products of fermentation. The hydrogen of the water and of the respiratory material is thereby bound to respiratory pigments, *i.e.*, oxidized chromogens. The first phase of respiration may be expressed by the following equation (since the chemical nature of the intermediate products of fermentation is unknown, the respiratory material is represented here by the empirical formula for sugar):



From this it is evident that all the CO_2 of oxygen respiration is of anaerobic origin, in Palladin's opinion. The carbon of the respiratory material is oxidized by the oxygen of water and of the respiratory material. The second phase of the oxidation processes of respiration consists in the oxidation of the respiratory chromogens by peroxidase at the expense of atmospheric oxygen (naturally through the intermediate stage of peroxide formation), whereby all the hydrogen taken from the respiratory material goes into water:



The hydrogen acceptors (chromogens) are thus completely regenerated and could be used in the oxidation of new portions of respiratory material. Thus according to Palladin, the atmospheric oxygen oxidizes only the labile hydrogen of the respiratory pigments. This process, taking place through the action of peroxidase, has nothing to do with the formation of CO_2 , a view which corresponds entirely with the current idea of the nature of the action of peroxidases. It has already been pointed out that peroxidase preparations seem to oxidize only phenol groups.

The assumption is certainly not precluded that the results of the above mentioned experiments of Kostychev, in which fermented sugar solutions develop CO_2 after the addition of peroxidase and hydrogen peroxide, are due to the presence in fermented sugar solutions of not only the respiratory material but also suitable hydrogen acceptors. These results can also be explained by Palladin's scheme.

Palladin has proved experimentally that the conversion of respiratory chromogens into pigments involves the absorption of oxygen from the surrounding air.¹ The above mentioned investigations of Wieland on acetic acid fermentation also favor Palladin's theory. The following interesting discovery of Oparin² can serve as a particularly clear illustration of Palladin's theory. From sunflower seeds Oparin isolated a substance which appears to be identical with chlorogenic acid and which brings about the oxidative deaminization of amino acids entirely in the sense of Palladin's scheme. The substance in question is changed to a green pigment as the result of the oxidation of its hydrogen. Chlorogenic acid $\text{C}_{16}\text{H}_{18}\text{O}_9$ is a depside and a constituent part of the tannin known as caffetannic acid.³ Caffetannic acid would thus be called a prochromogen.^y

Palladin's theory is an important working hypothesis which should be tested further.⁴

When we review the known facts in the field of the chemical processes which take place during oxygen respiration, we come to the conclusion that the experimental investigation is seeking absolutely new goals in recent years. Previously the principal object of investigation was merely the external side of respiration (influence of various irritant effects, of the stage of development, etc.). At present there is developing the real biochemistry of respiration which provisionally is recording only the isolated results, but the results already won allow the advancement of a coherent theory of respiration which can serve as a working hypothesis for further investigations. It is also evident now that the further development of the chemistry of plant respiration is closely bound with the chemical investigations of alcoholic fermentation. In the first place, it would be of the greatest importance to identify the compound which serves simultaneously as the intermediate product of oxygen respiration and of alcoholic fermentation, which is therefore formed from sugar through the participation

¹ Palladin and Tolstoi. *loc. cit.*

² Oparin, A. *Biochem. Zeitschr.* 124: 90. 1921; 182: 155. 1927.

³ Freudenberg, K. *Ber. chem. Ges.* 53: 232. 1920; Freudenberg und E. Vollbrecht. *Zeitschr. f. physiol. Chem.* 116: 277. 1921.

^y Still other respiratory chromogens or pigments have been isolated and studied within the last few years. Keilin (*Proc. Roy. Soc. London (B)* 98: 312. 1925; 100: 129. 1926; 104: 206. 1929) has described *cytochrome* which has been found in both animals and plants. It is a hydrogen acceptor which is said to be oxidized by "indophenol oxidase." For other work with cytochrome see also Shibata and Tamiya. *Acta Phytochim.* 5: 23-97. 1930. The colorless chromogen *hermidon* has been extracted from the leaves of *Mercurialis perennis* by Haas and Hill (*Biochem. Jour.* 19: 233. 1925; *Ann. Bot.* 39: 861. 1925; 40: 709. 1926) who have investigated the formation of colored pigments from it by oxidation. Haas and Hill also found a direct proportion between the amount of this chromogen present and the rate of respiration of the plant. From growing potato tubers Roach (*Ann. Bot.* 39: 882. 1925) has obtained a pigment which also acts something like Palladin's respiratory chromogens.—*Ed.*

⁴ On this point cf. Thunberg. *Skand. Arch. f. Physiol.* 30: 285. 1913; Meyerhof, O. *Pflügers Arch. f. ges. Physiol.* 170: 367 and 428. 1918; Vol. 175. 1919; *Idem.* *Zeitschr. f. physiol. Chem.* 101: 165. 1918; 102: 1. 1918.

of the enzymes of fermentation, and is then burned to the end-products of respiration by means of oxidizing agents.

The latest achievements in the field of the chemistry of fermentation and of plant respiration are also very interesting for chemistry in general because new types of chemical reactions have been discovered, which proceed most peculiarly and which have not been noticed before in organic chemistry.

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NOTE.—This index includes the names of authors cited in notes, the families and generic names of plants mentioned, and a large number of subject entries. In connection with these subject entries, incidental references to substances or processes have been largely omitted so that the entries indicate the points at which actual information is given. All authors' names are given in **bold-face** type and the pages on which they are followed by full citations are printed in the same type. The names of plants are placed in *italics*. All other entries are in ordinary type.—*Ed.*

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